

Muresk Institute

Effect of different ionic profiles of inland saline water on growth and agar characteristics of *Gracilaria cliftonii* (Withell, Miller & Kraft, 1994)

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Doctor in Philosophy
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DECLARATION

To the best of my knowledge and belief this thesis contains no material previously published by any other person except where due acknowledgment has been made.

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December 2008

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ABSTRACT

Salinity caused by the mobilisation of geologically stored salt through rising water tables is a major problem in Western Australia. Damage is occurring to aquatic and terrestrial ecosystems, affecting the profitability of agricultural land. However, the resultant inland saline water-bodies with an ionic composition similar to ocean water have the potential to be used for seaweed cultivation. Seaweed cultivation can mitigate the cost of land rehabilitation by making profitable use of saline wastewaters.

A series of laboratory trials were conducted to identify the conditions necessary for successful *Gracilaria cliftonii* cultivation in inland saline water (ISW) and modify the agar extraction process for *G. cliftonii* to optimise agar yield and properties. This was accomplished by culturing *G. cliftonii* with different nutrients in ISW, with or without K⁺ fortification and investigating the respective effects on chemical composition, physicochemical properties and agar characteristics. To identify the process required for optimum agar yield and quality from *G. cliftonii*, the effect of modifying some extraction process variables such as alkali concentration, soaking time and temperature, heating time, seaweed-water ratio, extraction time and temperature was investigated.

These trials demonstrated that with the selection of appropriate nutrient media and culture conditions *G. cliftonii* can be successfully grown in ISW. To achieve higher growth rates of *G. cliftonii*, it is necessary to fortify ISW with K⁺. The addition of nutrients to ISW and different ionic profiles of ISW had significant positive effects on chemical composition, physicochemical properties and agar characteristics. Agar yield, gel strength, melting point, gelling temperature and sulphate content were found to be a function of *G. cliftonii* life stages. Modification of alkali treatment with variable alkali concentration (0.3 to 5 %), soaking time and heating time had detrimental effect on agar yield resulting in significant agar loss but improved agar quality from *G. cliftonii*. However, agar quality can be improved by modifying extraction process variables like soaking time and temperature, seaweed to water ratio, extraction time and temperature without agar loss.

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LIST OF ABBREVIATIONS

ISW	Inland saline water
OW	Ocean water
AGR	Apical growth rate
SGR	Specific growth rate
WRC	Water retention capacity
ORC	Oil retention capacity
S.E.	Standard error
%db	Percentage dry basis
N/A	Not applicable
PES	Provasoli enriched seawater
USP	United States Pharmacopeia
ppt	Parts per trillion
temp.	Temperature

PREAMBLE

This thesis includes eight experiments in order to investigate the effect of different nutrient and ionic profiles of inland saline water on *G. cliftonii* growth parameters, chemical composition, physicochemical and agar properties. The thesis is divided into twelve chapters.

Chapter 1 begins with the history, production and utilisation of different seaweeds followed by the biology of *G. cliftonii*. This is followed by a review of the culture techniques and environment and role of nutrients in different *Gracilaria* species and osmoregulation in different seaweeds. This chapter reviews the extent and characteristics of ISW and culture of organisms in this medium. This is followed by a review of the chemical composition and physicochemical properties of different seaweed species. The chapter reviews the agar characteristics and extraction process for different *Gracilaria* species.

Chapter 2 introduces the research topic and justifies the research followed by the general methodology used throughout the research. Chapter 3 identifies the potential of *G. cliftonii* as a candidate species for ISW culture. This chapter includes the chemical composition, physicochemical properties and agar characteristics of different life stages of *G. cliftonii*. This chapter also identifies the cultivation technique to be employed for culturing *G. cliftonii*.

Chapter 4 incorporates two trials of six weeks duration. The first trial investigated the effect of four commercially available nutrient media (f2, Aquasol, Walnes and fertiliser) on the growth rate of *G. cliftonii* in ISW and OW in small volumes (500 mL). The second trial investigated the effect of three commonly used nutrient media (f2, Walnes and PES) in seaweed culture on the growth rate and net yield of *G. cliftonii* in ISW and OW in large volumes (25 L) and their effects on physicochemical properties and agar characteristics of *G. cliftonii*.

Chapter 5 investigates the effect of nutrient supplementation of ISW and OW on the growth rate, net yield, physicochemical properties and agar characteristics of *G. cliftonii*. In this trial, N, P and K were used in four different ratios of 1:1:1, 2:1:1, 1:2:1 and 1:1:2 as nutrient supplements for culturing *G. cliftonii* in ISW and OW for nine weeks.

Chapter 6 presents a trial to investigate the effects of different ionic profiles of ISW on the growth rate, chemical composition and agar characteristics of *G.*

cliftonii. In this trial different ionic profiles of ISW were achieved by mixing it with OW at salinities of 25 ppt and 35 ppt. The growth rates were monitored for six weeks then chemical composition and agar properties were determined at the end of trial.

Chapter 7 investigates the effect of potassium fortification of ISW on the growth rate, net yield, chemical composition, physicochemical properties and agar characteristics of *G. cliftonii*. In this trial the ionic profiles from chapter six were fortified with potassium to achieve an equal concentration at salinities of 25 and 35 ppt. The trial was conducted for three months with growth rates monitored monthly while chemical composition, physicochemical properties and agar characteristics were determined at the end of the trial.

Chapter 8 investigates the influence of alkali treatment, soaking and heating time on agar yield and properties (gel strength, gelling temperature, melting point and sulphate content) from different life stages of *G. cliftonii*. In this trial, reproductive and vegetative stages of *G. cliftonii* were treated with 0.3 and 0.5 % alkali with and without heating time and variable soaking time.

Chapter 9 and 10 include modification of the alkali treatment on *G. cliftonii* by varying alkali concentrations and other input variables to optimise agar yield and properties.

Chapter 11 includes modification of the overall agar extraction process of *G. cliftonii* to optimise the agar yield and properties. The final chapter (Chapter 12) discusses and compares results of all the trials conducted in this study. Further, conclusions are drawn from the main findings which are then followed by recommendations for further research.

CHAPTER 1: LITERATURE REVIEW

1.1. Seaweed

1.1.1. History

Seaweed has historically been defined in a variety of ways and its definition is still not clear. The term “seaweeds” generally includes only macroscopic, multicellular marine red, green and brown algae (Lobban and Harrison, 1994). Guiry (2008) defines seaweeds as “marine algae: saltwater-dwelling, simple organisms that fall into the rather outdated category of plants”. Seaweeds are classified according to their pigmentation into three main groups: Phaeophyta (brown algae), Rhodophyta (red algae) and Chlorophyta (green algae). Guiry (2008) has compiled about 1200 green species, 1750 brown species and 6000 red species on “Algaebase”. Seaweeds are important primary producers with worldwide distribution dominating rocky intertidal shores and temperate and shallow subtidal regions. They are found at depths of 8-35 m in most oceans, although some may be found to depths of 250 m (Lobban & Harrison, 1994).

The use of seaweed as food has been traced back to the fourth century in Japan and the sixth century in China. Today those two countries and the Republic of Korea are the largest consumers of seaweed as food (McHugh, 2003). However, as nationals from these countries have migrated to other parts of the world, the demand for seaweed as food has followed them, as, for example, in some parts of the United States of America and South America. Increasing demand over the last fifty years outstripped the ability to supply requirements from natural (wild) stocks. Research into the life cycles of these seaweeds has led to the development of cultivation industries that now produce more than 90 percent of the market's demand (McHugh, 2003). *Chondrus crispus* has traditionally been eaten in Ireland, Iceland and Nova Scotia (Canada) and this market is being developed. Markets also exist for seaweed in some developing countries where fresh seaweed is consumed as vegetables and in salads (McHugh, 2003).

1.1.2. Production

China is the largest producer of edible seaweeds, harvesting about 5 million wet tonnes. The greater part of this includes *Laminaria japonica* which is grown on suspended ropes in the ocean. The Republic of Korea grows about 800,000 wet tonnes of three different species, about 50 percent of which is *Undaria pinnatifida*,

grown in a similar fashion to *Laminaria* in China. Japanese production is around 600,000 wet tonnes, 75 percent of which is *Porphyra* species, the thin dark seaweed wrapped around a rice ball in sushi. It is a high value product, about USD 16,000/dry tonne, compared to *Laminaria* at USD 2,800/dry tonne and *Undaria* at USD 6,900/dry tonne (McHugh, 2003).

Zemke-White & Ohno (1999) reported that in 1994/1995 over 2 million tonnes dry weight of seaweed was produced annually out of which 90% came from just six countries (China, France, UK, Korea, Japan and Chile) and just five genera (*Laminaria*, *Lithothamnion*, *Porphyra*, *Undaria* and *Gracilaria*). Out of this, 52% of seaweed produced was cultured which included 74% of chlorophytes, 22% of rhodophytes and 82% of phaeophytes. China, Korea and Japan produced 90% of the cultured seaweed. *Laminaria* had the largest production (682,581 tonnes dry wt.) followed by *Porphyra* (130,614 tonnes dry wt), *Undaria* (101,708 tonnes dry wt.) and *Gracilaria* (50,165 tonnes dry wt.). These four genera made up 93% of seaweed cultured worldwide (Zemke-White & Ohno, 1999). The annual *Gracilaria* production is shown in Table 1.1.

Table 1.1: Annual *Gracilaria* production 1994-95 (tonnes dry weight)

Country	Total	Cultured
Argentina	2276	N/A
Chile	68436	34218
China	300	300
India	215	N/A
Indonesia	13447	13447
Mexico	205	N/A
Thailand	200	200
USA	2	N/A
Vietnam	2000	2000

Source: Modified from (Zemke-White & Ohno, 1999)

1.1.3. Utilisation

Seaweeds have many uses but they are mainly used as food and as source of raw material for the extraction of phycocolloids (i.e. agar, carrageenan, and alginate).

Red and brown seaweeds are used to produce three hydrocolloids. Agar and carrageenan are extracted from red seaweeds and alginate from brown. A hydrocolloid is a non-crystalline substance with very large molecules and which dissolves in water to give a thickened (viscous) solution. Alginate, agar and carrageenan are water-soluble carbohydrates that are used as thickening, emulsifying, stabilizing and jellifying agents in food and pharmaceutical industries such as ice cream (they inhibit the formation of large ice crystals so that the ice cream can retain a smooth texture) (McHugh, 2003).

Zemke-White (1999) reports the total phycocolloid production in three different categories in 1994-95 was 108,229 tonnes (dry wt.) of agarophytes (i.e. *Gracilaria*, *Gelidium*), 81,858 tonnes (dry wt.) of carrageenophytes (i.e. *Kappaphycus*, *Chondrus*) and 826,178 tonnes (dry wt.) of alginophytes (i.e. *Sargassum*). The phycocolloid quantity cannot be precisely estimated, as phycocolloid content varies between different species, seasonally and among species at different locations. According to McHugh (2003) approximately 1 million tonnes of wet seaweed are harvested and extracted annually to produce the above three hydrocolloids. Zemke-White (1999) reported an estimated 25% yield from agarophytes would give a total possible worldwide yield of 27,057 tonnes agar. Using USD 10/kg for agar the phycocolloid production would have an approximate annual value of USD 2.6 billion.

An estimation of the economic value of seaweeds is difficult to gather because of competition, farmers and harvesters being loath to give accurate figures on the price paid for their products and limited published material (Zemke-White & Ohno, 1999). With the exception of the highly organized industry in Asia, much of the seaweed gathered for food is done by cottage industry or local families and is generally not regulated nor is production recorded (Zemke-White & Ohno, 1999).

1.2. *Gracilaria cliftonii*

1.2.1. Distribution

Seven species of *Gracilaria* have been identified in Australia, five of which are found in Western Australia: *Gracilaria cliftonii*, *G. blodgettii*, *G. perissana*, *G. canaliculata* and *G. flagelliformis* (Huisman, 2000). These species are distributed along Western Australian's coastline around Perth, Walkerville in Victoria, and

around Tasmania (Withell *et al.*, 1994). *G. cliftonii* is found from Geraldton to Esperance and is high in agar content (Byrne *et al.*, 2002) (Plate 1.1).

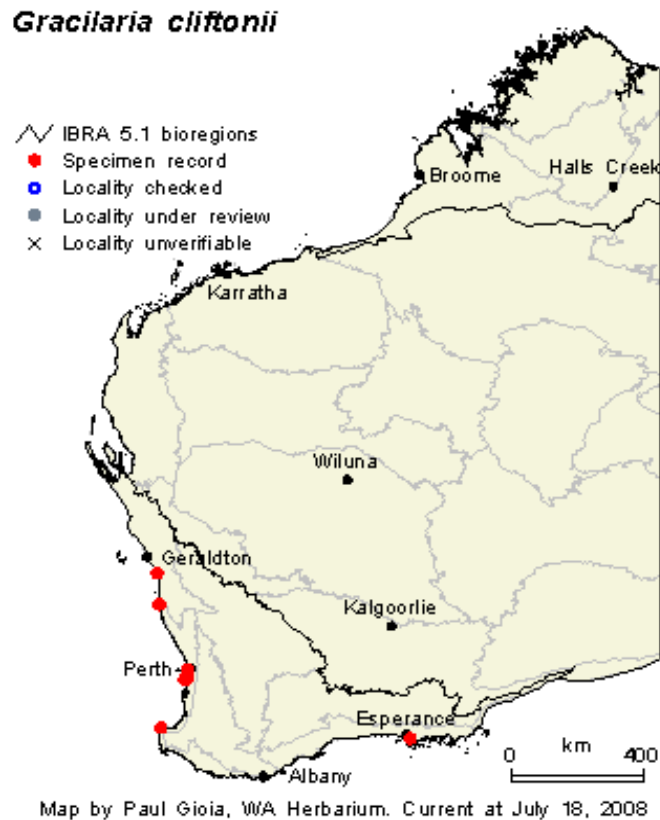


Plate 1.1: Distribution of *G. cliftonii* in Western Australia

1.2.2. Morphology and Vegetative anatomy

The morphology of *G. cliftonii* as described by Byrne *et al.* (2002) presents burgundy to dark red thalli, 20–25 cm high and is cartilaginous and terete throughout. It is attached to rocks by a holdfast. Thallus are terete and rise either singly or in dense clusters from the holdfast. Plants arising from individual holdfasts are usually robust while thalli from consolidated holdfasts are usually of lesser height and diameter. Liquid-preserved specimens are firm and pliable in texture while dried specimens are firm and brittle. Byrne *et al.* (2002) described the vegetative anatomy of *G. cliftonii* as a gradual transition of cells from cortex-to-medulla, with cells increasing to 250 µm in diameter towards the frond centre. They illustrated the cortex as two to four cells thick with surface cortical cells being 12.5 µm wide and 12.5 µm long.

1.2.3. Life cycle

The *Gracilaria* life cycle is described as a “*Polysiphonia* type”, with 3 life stages, gametophyte, carposporophyte and tetrasporophyte (Plate 1.2). Gametophytes of *Gracilaria* are heterothallic, where male and female thalli are found on separate individuals. The male thalli produce spermatangia. The female gametophyte is fertilized in situ by the male gametes and develop into carposporophytes, which produce carpospores. The carpospores develop into tetrasporophytes. Tetraspores are produced from the tetrasporophytes and germinate into male and female gametophytes completing the cycle (UNDP/FAO, 1990).

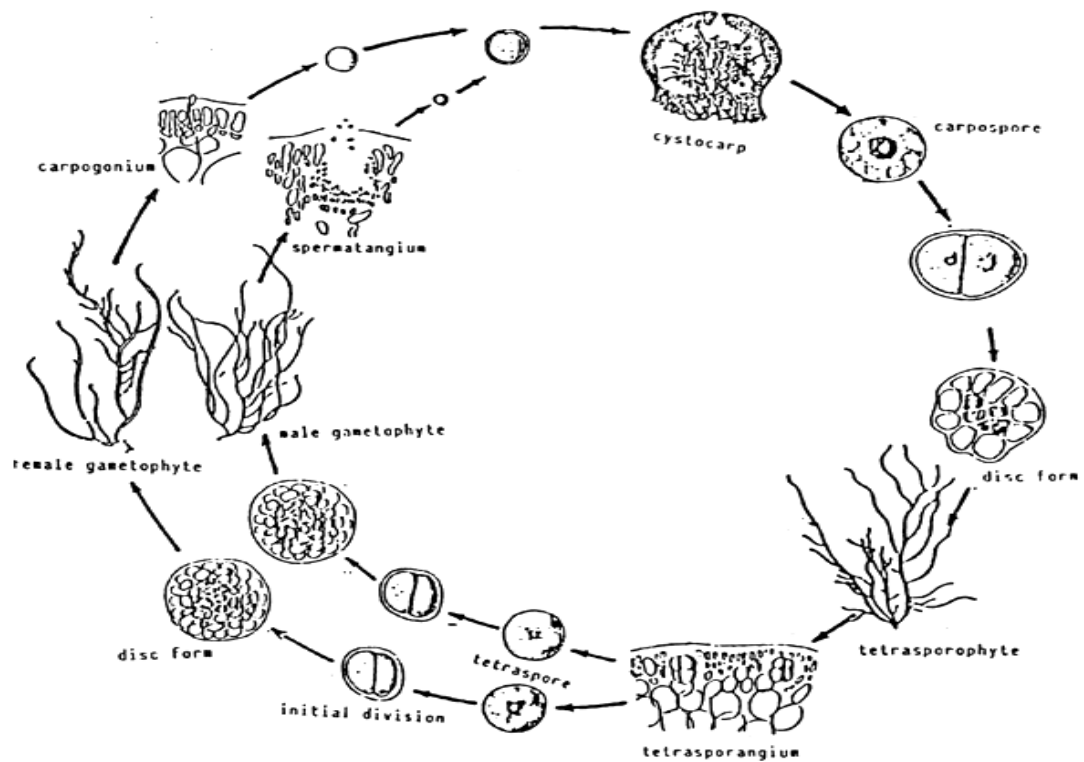


Plate 1.2: Life cycle of *Gracilaria* species (UNDP/FAO, 1990)

1.2.3.1. Tetrasporophyte

As described by Withell *et al.* (1994) tetrasporangia are divided cross-shaped (cruciate) with a length of 29-48 μm and width of 20-27 μm and are embedded in branches in the upper half of the plant. The cortex surrounding mature tetrasporangia consists of 3-5 cell layers with the cells frequently larger and more elongate than cells in vegetative portions of the plant.

1.2.3.2. Gametophyte

The spermatangia structure is obovoid to globose, 3-5 µm long and 3-4 µm in diameter. It is embedded in ovoid verrucosa-type conceptacles or very rarely in deep, cavern-like polycavernosa-type conceptacles. The surrounding cortical cells frequently elongate compared with cortical cells in vegetative portions of the plant. Conceptacles are 55-100 µm deep by 30-50 µm across and are continuous when mature and borne on the entire frond except basal and extreme apical portions (Withell *et al.*, 1994).

1.2.3.3. Carposporophyte

Carpogonial branches are present in the cortex of younger branches, borne on subcortical cells. Mature carpogonial branches are two-celled, the hypogynous cell is transversely ovoid to subspherical and the carpogonium conical to ovoid. As the fusion cell forms, the layers of the cortical cells divide both anticleinally and pericleinally to form the pericarp. Mature cystocarps ostiolate, 0.7-1.2 mm high by 1.0-2.0 mm wide, occasionally basally constricted and/or beaked; pericarp 200-420 µm thick, consisting of 12-18 cell layers; gonimoblast parenchyma moderately abundant, the basal cells 75-90 µm long by 43-55 µm wide. Carpospores ovoid to obovoid, 32-56 µm long by 16-32 µm diameter, containing a central chloroplast and borne terminally on the gonimoblast filaments (Withell *et al.*, 1994).

1.3. Gracilaria cultivation

Commercial cultivation of *Gracilaria* species began in the late 1980's because of the over-exploitation of wild stocks. At present 80-90 % of *Gracilaria* species production is through cultivation of wild stocks (Alveal *et al.*, 1997). *Gracilaria* species can be cultivated from spores or through vegetative propagation (Alveal *et al.*, 1997; Friedlander & Dawes, 1984a; Glenn *et al.*, 1996; McLachlan & Bird, 1986; Santelices & Doty, 1989; Ugarte & Santelices, 1992). A number of methods have been developed for the cultivation of *Gracilaria* species. Some seaweed can be cultivated vegetatively while others only by going through a separate reproductive cycle, involving alternation of generations. The vegetative method is mostly used where cultivation is used to produce seaweeds for the hydrocolloid industry (agar and carrageenan), while the principal seaweeds used as food must be taken through the alternation of generations for their cultivation. The most commonly used methods for vegetative cultivation of *Gracilaria* are bottom planting, line farming, raft cultivation and suspended farming (Santelices & Doty, 1989). The

growth rates and net yield for different species with different cultivation methods are shown in Table 1.2.

1.3.1. Bottom planting

In the bottom planting cultivation technique of *Gracilaria* species, vegetative fronds are grown in tanks or field with non-consolidated material at the bottom i.e. sand, mud, sediments, shells etc. The seaweed is harvested when it is 10-15 cm high. The depth for submerging the seaweed ranges from 0.5 m to 1.5 m. Planting and harvesting are high labour-consuming activities, which can be economic only in areas with low labour costs (Pizarro & Barrales, 1986; Santelices & Doty, 1989).

1.3.2. Line farming

In the line farming cultivation technique, large cuttings or whole thalli (2-5 cm) are attached to ropes manually and then submerged in seawater in the tank or field. Nylon or coir ropes are used for anchoring the thalli. The seaweed is harvested when it is 10-15 cm high by cutting the thalli, leaving 1-2 cm on the rope, for propagation of the next crop (Alveal *et al.*, 1997; Santelices & Doty, 1989).

1.3.3. Raft farming

In raft farming, fragments of *Gracilaria* species obtained from the wild crops are attached to ropes each about 70 cm long. These are hung horizontally on low fixed rafts or vertically on floating rafts placed in tanks (Santelices & Doty, 1989).

1.3.4. Suspended farming

Suspended cultivation methods, using vegetative thalli attached to lines, have been extensively developed along Asian and Atlantic temperate coasts. Studies have concerned mainly the effect of nutrient addition, water movement, light intensity, epiphyte interference and diverse abiotic factors on the growth rate and yield of *Gracilaria* (Capo *et al.*, 1999; Doty & Fisher, 1987; Thomas & Krishnamurthy, 1976).

Table 1.2: Growth rate and yield of *Gracilaria* species with different cultivation techniques

SPECIES	Cultivation Technique	Inoculum Kgm ⁻²	Growth rate	Yield
				0.2-2.4
<i>G. chilensis</i> ¹	Suspended	0.6	N/A	Kg.m ⁻²
	Rope	1.2		0.2-1.8
				Kg.m ⁻²
<i>G. chilensis</i> ²	Ropes	200g in 340L	N/A	2-15 kg.m ⁻¹
<i>G. parvispora</i> ³	Bottom planting/line	2.22	1.22-4.52 cmweek ⁻¹	126-974 g.m ⁻²
			20-60 %day ⁻¹	
<i>G. tikvahiae</i> ⁴	Spore culture	0.4-4.8	1-5 %day ⁻¹	2-3
			19-60 %week ⁻¹	
<i>G. conferta</i> ⁵	Spore culture	0.8		N/A
<i>G. corticata</i> ⁶	Laboratory culture	2cm thalli	Double in 25days	N/A
<i>G. heteroclada</i> ⁷	Vegetative/ rice planting method	15-20 g thalli	N/A	7-14 tha ⁻¹ yr ⁻¹
<i>G. lemaneiformis</i> ⁸	Raft / line rope Vegetative	25-200 g.m ⁻¹	13.9 %day ⁻¹	1256 gm ⁻¹
<i>G. chilensis</i> ⁹	Tank	4, 6, 8	N/A	8-120 g.m ⁻² day ⁻¹

Source: 1.(Westermeyer *et al.*, 1993), 2.(Alveal *et al.*, 1997), 3.(Glenn *et al.*, 1996), 4.(Lapointe & Ryther, 1978), 5.(Friedlander & Ben-Amotz, 1991), 6.(Subbaraju *et al.*, 1981), 7.(Hurtado-Ponce *et al.*, 1992), 8.(Yang *et al.*, 2005), 9. (Ugarte & Santelices, 1992)

1.4. Culture Environment

In the last three decades, different authors have reported different culture variables to manipulate and control the growth of *Gracilaria* species. The major variables affecting growth include light, temperature, salinity, pH and nutrients (Choi *et al.*, 2006; Daugherty & Bird, 1988; Dawes *et al.*, 1998; Ekman *et al.*, 1991; Friedlander & Dawes, 1984b; Hong *et al.*, 2007; Israel *et al.*, 1999). Light is the most important but complex factor affecting seaweeds. The primary importance of light to seaweeds is in providing the energy for photosynthesis (Hong *et al.*, 2007; Lobban & Harrison, 1994). Temperature is the second important physical factor for organisms as it affects all levels of biological organisation. High temperature results in denaturation of proteins and damage to enzymes and membranes while low temperature can cause disruption of the lipids and proteins in membranes and mechanical damage to the cell through the production of ice crystals (Lobban & Harrison, 1994). The next most important parameter is salinity as it is responsible for osmotic consequences of the movement of water molecules, along the water molecules, along water-potential gradients and the flow of ions along electrochemical gradients (Choi *et al.*, 2006; Daugherty & Bird, 1988; Ekman *et al.*, 1991; Israel *et al.*, 1999). These processes take place simultaneously and both are regulated in part by semi-permeable membranes that surround cells, chloroplasts, mitochondria and vacuoles (Lobban & Harrison, 1994). The pH of seawater remains basic ranging between 7.4 and 8.4 due to carbonate buffering (Lobban & Harrison, 1994) and has been altered in culture conditions to investigate its effect on growth and agar characteristics of different *Gracilaria* species. Numerous studies have been performed on *Gracilaria* species, to investigate the effect of light, temperature, salinity, pH and nutrients on growth and agar characteristics in natural populations and under culture conditions (Choi *et al.*, 2006; Daugherty & Bird, 1988; Dawes *et al.*, 1998; Ekman *et al.*, 1991; Friedlander & Dawes, 1984b; Hong *et al.*, 2007; Israel *et al.*, 1999) and are summarised in Table 1.3.

Table 1.3: Culture conditions and environment used for cultivating *Gracilaria* species

Species	Condition	Light	Temperature	Salinity	pH	Nutrient
<i>G. tikvahiae</i> ¹	Outdoor	Yes	No	No	No	Yes, NO ₃ ⁻
<i>G. parvispora</i> ²	Field, water quality factors	No	Yes	Yes	No	Yes, NO ₃ ⁻ PO ₄ ⁻
<i>G. tenuistipitata</i> ³	Ponds, Seasonality	No	Yes	No	No	No
<i>G. tenuistipitata</i> ⁴	Laboratory, Outdoor tanks	Yes	Yes	Yes	Yes	Yes
<i>G. chorda</i> ⁵	Field, Indoor tanks	Yes	Yes	No	No	Yes, metal ions
<i>G. parvispora</i> ⁶	Field, cage culture	No	No	No	No	Yes, NH ₄ ⁺ , PO ₄ ⁻
<i>G. tikvahiae</i> ⁷	Outdoor culture	Yes	No	Yes	No	No
<i>G. cornea</i> ⁸	Field, Seasonality	No	Yes	No	No	No
<i>G. conferta</i> ⁹	Outdoor tanks	Yes	Yes	No	No	Yes, N
<i>G. conferta</i> ¹⁰	Outdoor tanks	Yes	Yes	Yes	Yes	Yes, NH ₄ ⁺ , NO ₃ ⁻ PO ₄ ⁻

Source: 1.(Lapointe & Duke, 1984), 2.(Glenn *et al.*, 1999), 3.(Lee *et al.*, 1999), 4. (Israel *et al.*, 1999), 5.(Kakita & Kamishima, 2006), 6. (Nagler *et al.*, 2003), 7.(Lapointe *et al.*, 1984), 8.(Orduña-Rojas & Robledo, 2002), 9.(Friedlander, 1991), 10. (Friedlander & Ben-Amotz, 1991)

Some of the other factors which have been studied under culture conditions and have been reported to affect the growth of *Gracilaria* species are water motion (Glenn *et al.*, 1999; Ryder *et al.*, 2004), water exchange (Buschmann *et al.*, 1994; Friedlander & Ben-Amotz, 1991), acclimation time (Gomez *et al.*, 2005; Israel *et al.*, 1999) and photoperiod (Kakita & Kamishima, 2006).

1.4.1. Nutrient Requirements

Nutrient supply is one of the most important operating parameters in the management of seaweed cultivation systems (Lignell & Pedersén, 1987). Seaweed nutrition (i.e. *Gracilaria* sp.) usually includes nitrogen and phosphate pulse feeding and is associated with increase of growth rates (apical and specific) and high yields (Capo *et al.*, 1999; Lapointe, 1985; Navarro-Angulo & Robledo, 1999; Smit *et al.*, 1996). In addition, it is reported that *Gracilaria* sp. have a higher absorption rate for NH_4 than for NO_3 since NH_4 is directly incorporated into the amino acid pool, resulting in better growth rates (Haglund & Pedersén, 1993; Lobban & Harrison, 1994). It is considered that the requirements of all algae are similar but the variability of macroalgae makes it difficult to specify their nutritional requirements. Some of the probable functions of elements in seaweeds are summarized in Table 1.4.

Table 1.4: Probable functions of some common elements in seaweed

Element	Probable functions
Nitrogen	Major metabolic importance in compounds
Phosphorus	Structural, energy transfer
Potassium	Osmotic regulation, pH control, protein formation and stability
Calcium	Structural, enzyme activation, cofactor in ion transport
Magnesium	Photosynthetic pigments, cofactor in ion transport
Sulphur	Active group in enzymes and coenzymes, structural
Iron	Active groups in porphyrin molecules and enzyme
Manganese	Electron transport, maintenance of chloroplast
Molybdenum	Nitrate reduction, ion absorption
Sodium	Enzyme activation, water balance
Chlorine	Photosystem, secondary metabolites

Source: Modified from (Lobban & Harrison, 1994)

1.4.1.1. Nutrients uptake and storage

Bird *et al.* (1982) examined internal nitrogen pools in thalli of *Gracilaria tikvahiae* as a function of total nitrogen content of the thallus, nitrogen deprivation, and nitrogen resupply. They found that amino acids and proteins form major nitrogen storage pools in *G. tikvahiae*. In addition, they found inorganic nitrogen in the forms of NH_4^+ and NO_3^- in the thalli but with minimal contribution to the total nitrogen pool. They reported that within the protein pool, the phycoerythrin pigments appeared important as a source of nitrogen when thalli were initially becoming nitrogen limited. In general, an inverse relationship between the levels of nitrogen and the carbohydrate content of the algal thalli was observed.

Naldi and Wheeler (1999) studied the accumulation of nitrogen in different cellular pools by the macroalgae *Gracilaria pacifica* (Abbott) (Rhodophyta) in a laboratory experiment. After 8 or 9 days of nitrogen enrichment, they extracted and analysed nitrate, ammonium, free amino acid (FAA), protein, chlorophyll (chl), phycoerythrin (PE), and insoluble nitrogen pools and their relative contribution to total nitrogen (TN). They found that in *G. pacifica*, TN increased more under ammonium enrichment than under nitrate enrichment with proteins and FAA as the most important N storage pools. They found that Protein-N contributed 43-66 % and the FAA pool accounted for 4-17 % of TN.

Lapointe and Ryther (1978) conducted series of outdoor, continuous-flow seawater cultures (50 L; 0.23 m^2) to investigate the effects of nutrient loading (total nitrogen input/day) with both NH_4^+ -N and NO_3^- -N on growth and yield of *Gracilaria tikvahiae*. They found that above a minimal daily nitrogen loading, the yield of *Gracilaria* was independent of nutrient concentration, nitrogen loading, and nitrogen source (NH_4^+ -N or NO_3^- -N).

Fujita (1985) investigated the NH_4^+ uptake kinetics of *Gracilaria tikvahiae* immediately after collection from the field, after acclimation to high and low nitrogen fluxes and after nitrogen starvation. He reported that transient uptake by algae grown at low N flux or starved for N was more rapid than uptake by algae grown at high N flux. In addition, uptake did not always follow Michaelis-Menten kinetics and was often a linear function of concentration at higher concentrations. He concluded that high uptake rates during periods of high N availability could be important in supporting N-limited growth in environments with episodic N supply.

Naldi and Wheeler (2002) determined ammonium and nitrate uptake rates in *Gracilaria pacifica* (Abbott) (Rhodophyta) by ^{15}N accumulation in algal tissue and by disappearance of nutrient from the medium in long-term (4–13 days) incubations. They found uptake of NH_4^+ was highest on the first day of incubation (32 pmol N.g.dwt $^{-1}$.h $^{-1}$) with negligible uptake of NO_3^- during the first 2–3 days. They reported that lag of uptake may have resulted from the need for either some N deprivation (use of NO_3^- pools) or physiological/metabolic changes required before the uptake of NO_3^- .

Smit (2002) investigated the physiology of nitrogen acquisition by *Gracilaria gracilis* in a series of experiments to examine uptake kinetics in response to variable nitrogen. He found that ammonium-nitrogen uptake was linear, rate-unsaturated response, with the slope increasing with N-limitation, which indicated that *Gracilaria* is more efficient at acquiring nutrients when internally stored N pools were low. In addition, he found that nitrate-nitrogen uptake was suppressed by approximately 38 % in the presence of NH_4^+ -N at concentrations above 5 μM , and seaweed showed a higher affinity for NH_4^+ -N than for NO_3^- -N at low temperatures. Increased temperature improved the affinity for NO_3^- -N only in N-limited thalli, while nutrient limitation enhanced affinity irrespective of temperature.

Lapointe (1981) studied combined effects of light intensity and nitrogen (NO_3^-) on growth rate, pigment content, and biochemical composition of *Gracilaria foliifera* using outdoor continuous cultures. He found that growth of *Gracilaria* increased linearly with increasing light to 0.43 doublings d $^{-1}$ at high light levels (383 ly.d $^{-1}$ of in situ light), suggesting that light may often limit growth of this plant in nature. In addition, Chlorophyll a and phycoerythrin contents were inversely proportional to light level and growth rate. However, pigment content did not affect the growth capacity of *Gracilaria* and there was no increase in growth or pigment content with increasing additions of nitrogen. He also reported that faster growing plants had C:N ratios above 10, indicating N- limitation.

Friedlander and Dawes (1985) studied the uptake kinetics of ammonium and phosphate by *Gracilaria tikvahiae* under field conditions. They found that seaweeds, pulse fed once a week for 6 h over a 4-week period had maximum uptake rates of 19 $\mu\text{mol.g.fwt}^{-1}$.h $^{-1}$ for NH_4^+ and 0.28 $\mu\text{mol.g.fwt}^{-1}$.h $^{-1}$ for PO_4^- . In addition, both nutrients showed positive linear correlation between uptake rate and concentration.

Tsai *et al.* (2005) investigated the effects of temperature and nutrient availability on seasonal growth dynamics of *Gracilaria coronopifolia*. They observed year round appearance of *G. coronopifolia* with tolerance to wider temperature range and a significant stimulation of growth by high N inputs. They also found that growth of *G. coronopifolia* was P-limited which was indicated by increased tissue N contents along with decreased tissue P contents. Significant drops in tissue P contents reveal severe P limitation, which is due to increased alkaline phosphatase activity.

1.4.1.2. Osmoregulation

Osmosis may be defined as the diffusion of a solvent through a semi-permeable membrane because of a concentration gradient, i.e. from a region of high to one of lower concentration of the solvent (Hellebust, 1976). The osmotic pressure (osmotic value or potential) of a solution may be defined in terms of measurement by an osmometer. Osmotic pressure is a measure of the tendency of water to move into one solution from another by osmosis (Hellebust, 1976). The higher the osmotic pressure of a solution the more water wants to go into the solution. Pressure must be exerted on the hypertonic side of a selectively permeable membrane to prevent diffusion of water by osmosis from the side containing pure water (Zimmermann, 1978). Osmoregulation is the active regulation of the osmotic pressure of bodily fluids to maintain the homeostasis of the body's water content; that is it keeps the body's fluids from becoming too dilute or too concentrated (Zimmermann, 1978).

Dissolved salts have two kinds of effects upon aquatic organisms. The first is related to the specific chemical nature of the ions in the solution and to the specific actions on living cells. The second, the osmotic effect, depends essentially on the total number of dissolved particles, and directly influences the movement of water into or out of the cells as if the latter were semi-permeable vesicles (Guillard, 1962).

Ion relations in marine algae were investigated intensively in mid 1950's to late 1960's, especially with respect to active or passive ion uptake to explain the different ion composition of sap and sea water but there is not much literature for recent developments (Guillard, 1962; Hellebust, 1976; Zimmermann, 1978). Active transport between two phases is specified by measuring ion concentrations in the two phases, potential difference and ion flux between the two phases (Graves & Gutknecht, 1976; MacRobbie & Dainty, 1958; Raven, 1967; Reed *et al.*, 1981;

Ritchie, 1985). The published literature on ionic relations in different seaweed species are summarized in Table 1.5.

Table 1.5: Seaweed species and variables studied for ion regulation

SPECIES	VARIABLES	IONS	REFERENCE
<i>Ulva lactuca</i>	Ion regulation mechanism	Na^+, K^+	(Scott & Hayward, 1953a)
<i>Ulva lactuca</i>	Light, temperature, ion exchange	K^+	(Scott & Hayward, 1953b)
<i>Ulva lactuca</i>	Metabolic factors, ion distribution	Na^+, K^+	(Scott & Hayward, 1953c)
<i>Ulva lactuca</i>	Membrane potential	Na^+, K^+	(Reed & Collins, 1981)
<i>Enteromorpha intestinalis</i>	Energetic considerations, Ion transport	$\text{Na}^+, \text{K}^+, \text{Cl}^-$	(Ritchie, 1985; Ritchie & Larkum, 1984a; b)
<i>Porphyra purpurea</i>	Membrane potential	Na^+, K^+	(Reed & Collins, 1981)
<i>Porphyra purpurea</i>	Ionic relations	$\text{Na}^+, \text{K}^+, \text{Cl}^-$	(Reed & Collins, 1980)
<i>Porphyra perforata</i>	Ion movement	$\text{Na}^+, \text{K}^+, \text{Rb}^+, \text{Cl}^-$	(Eppley, 1958a)
<i>Porphyra perforata</i>	Ion movement	$\text{Na}^+, \text{K}^+, \text{Ca}^{2+}, \text{Cl}^-$	(Eppley, 1958b)
<i>Gracilaria foliifera</i>	Ion distribution and transport	$\text{Na}^+, \text{K}^+, \text{Rb}^+, \text{Cs}^+, \text{Cl}^-, \text{Br}^-, \text{I}^-$	(Gutknecht, 1965)
<i>Gracilaria chorda</i>	Effect on growth	$\text{Fe}^{2+/3+}, \text{Co}^{2+/3+}$	(Kakita & Kamishima, 2006)

Table 1.5 shows the ion transport and ionic relations studied in various seaweeds. However, the transport of ions has been studied only through alteration of salinity and their role in growth rate has been poorly reported (Choi *et al.*, 2006; Ekman *et al.*, 1991; Lapointe *et al.*, 1984). Prangnell (2006) and Tantulo (2007) recently reported that different ionic profiles of ISW result in the movement of ions in prawns but there is no published information for similar phenomenon in seaweeds.

1.5. Inland Saline Water Aquaculture

1.5.1. Introduction

The use of inland saline water to culture marine species has been practiced widely in other countries. The potential for inland production of marine fish is now becoming recognized in Australia including South Australia. Salinity caused by the mobilisation of geologically stored salt through rising water tables is a major problem in Western Australia. Serious damage is occurring to aquatic and terrestrial ecosystems, affecting the profitability of agricultural land (Bailey *et al.*, 1997). An estimated 4.3 million hectares (16%) of the southwest region of Western Australia has a high potential of developing salinity from shallow water tables. This is predicted to rise to 8.8 million hectares (33%) by 2050 (Anonymous, 2001).

1.5.2. Causes of saline groundwater

Rising saline groundwater is becoming a major agricultural problem in many parts of South Australia. It occurs when vegetation is cleared from the surrounding land causing groundwater to rise and increase in salinity (Plate 1.3).

This is mainly due to shallow-rooted crops using less rainfall than deep-rooted native vegetation therefore reducing the quantity of rainfall being used in that area by the plants. Water not taken up by plants will seep into the watertable thus causing it to rise. The water then becomes saline through salts leeching in from the surrounding sediments and rock strata.

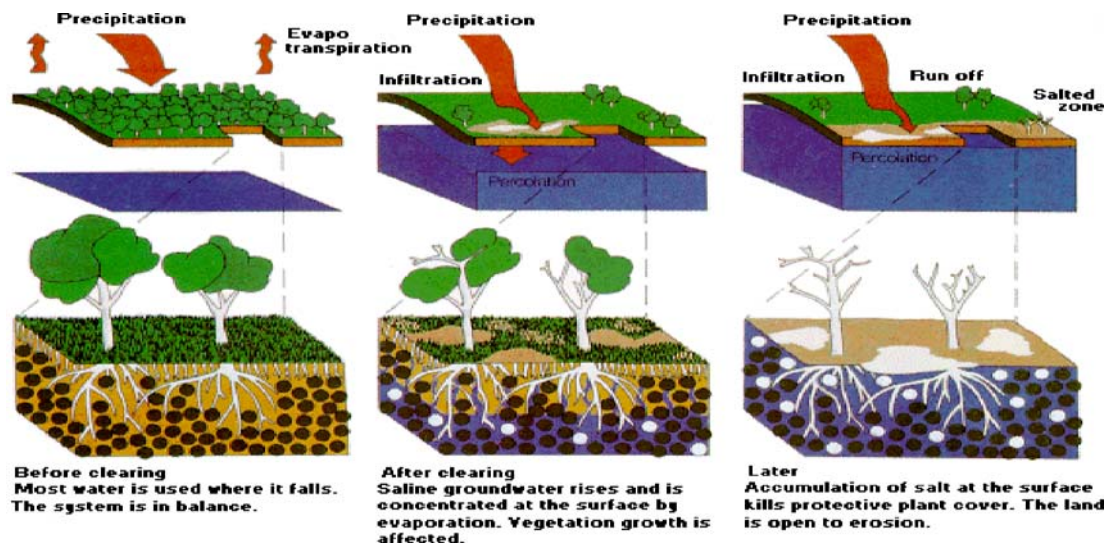


Plate 1.3: Causes of dryland salinity

1.5.3. Remedy

Disposal evaporation basins have been shown to be the least-cost option for removing high salinity drainage water and have become necessary to protect productive farmland, town infrastructure and downstream water supplies from rising saline water tables. These evaporation basins take further land out of production and incur operation costs for pumping and drainage without providing any productive return from land used for basins to the farmers and irrigation companies (Cordover, 2004).

Saline ground water and evaporation basins can be considered as a resource as well as an environmental expense (Cordover, 2004). These inland saline waterbodies with an ionic composition similar to ocean water can have potentially productive use for cropping appropriate seawater plants such as seaweeds.

Table 1.6: Locations around the world where potassium deficiency in inland saline water has been reported

COUNTRY	LOCATION
Australia	New South Wales Queensland South Australia Victoria Western Australia
China	
Ecuador	
India	
Israel	
USA	Alabama Arizona Arkansas Mississippi Texas

Source: Modified from (Prangnell, 2006)

1.5.4. International research and production

Inland saline water aquaculture at a research or commercial stage around the world is expanding to several species of algae, molluscs, crustaceans and finfish. A few of these species are summarised in Table 1.7.

Table 1.7: Species grown in inland saline water/ saline groundwater around the world, at research stage (R) or commercial production stage(C)

SPECIES	COUNTRY	STAGE
Micraolage	Israel, USA	R,C
Marine diatom	USA	R
Manila clam	France	R
Oysters	USA	R,C
American lobster	USA	R
Brine shrimp	USA	R,C
Hermit crab	USA	R
White shrimp	USA	R
Channel catfish	USA	C
Common carp	India	R
Common sole	Egypt	R
Eels	USA	R
Milkfish	India	R
Mullet	Egypt, India	R, C
Pearl spot	India	R
Rainbow trout	USA	R
Red drum	USA	R
Seabass	India	R
Tilapia	Israel	R

Source: Modified from (Prangnell, 2006)

1.5.5. Australian research and production

Australian farmers affected by increasing salinity are finding that investment in inland aquaculture provides the benefits of rehabilitating salt degraded land in addition to a valuable sideline to their business. Research into rearing various marine and salt tolerant fresh water species in ISW has been conducted in all mainland Australian states with small-scale production of some species progressing. The species grown in ISW around Australia are summarised in Table 1.8.

Table 1.8: Species grown in inland saline water around Australia, at research stage (R) or commercial production stage(C)

SPECIES	STATE/S	STAGE
<i>Dunaliella salina</i>	NT, SA, WA	R,C
Greenlip abalone (<i>Haliotis laevis</i>)	WA	R
Pacific oyster (<i>Cassostrea glomerata</i>)	Vic	R
Trochus (<i>Trochus niloticus</i>)	NT	R
Brine shrimp (<i>Artemia salina</i>)	SA, Vic	R,C
Banana prawns (<i>Penaeus merguensis</i>)	Qld.	R
Kuruma prawns (<i>P. japonicas</i>)	Vic.	R
Tiger prawns (<i>P. monodon</i>)	NSW, NT, Qld., Vic., WA	R
Western King Prawns (<i>P. latisulcatus</i>)	WA	R
Western Rock Lobsters (<i>Panulirus cygnus</i>)	WA	R
Atlantic Salmon (<i>Salmo salar</i>)	Vic	R
Barramundi (<i>Lates calcarifer</i>)	NSW, WA, SA	R,C
Black bream (<i>Acanthopagrus butcheri</i>)	SA, Vic, WA	R,C
European carp (<i>Cyprinus carpio</i>)	Vic	R
Mulloway (<i>Argyrosomus hololeptidotus</i>)	SA, WA, NSW	R,C
Rainbow trout (<i>Oncorhynchus mykiss</i>)	NSW, Vic, WA	R,C
Silver Perch (<i>Bidyanus bidyanus</i>)	NSW, SA, Vic	R, C
Snapper (<i>Pagrus auratus</i>)	NSW, WA, Vic, SA	R,C
Yellow fin whiting (<i>S. schomburgkii</i>)	SA	R

Source: Modified from (Prangnell, 2006)

The majority of the farmers interviewed by Cordover (2004) considered fish and prawns to be already troublesome under normal aquaculture practices and too financially risky to trial as a new crop. The major concerns were high infrastructure cost, labour requirements, post-harvest handling and transportation of seafood. Seaweed as a saline crop appeared to be more suitable for farming as it involves the same techniques and experience already developed for cropping and husbandry. The cultivation, harvest, drying and bailing of seaweed is parallel to the making of hay. Seaweed is a crop that can mitigate the cost of land rehabilitation by making profitable use of saline wastewaters (Cordover, 2004).

1.6. Chemical Composition and Physicochemical Properties

The nutritional properties of all species of seaweeds are not completely known yet and they are usually estimated from their chemical composition (Darcy-Vrillon, 1993; Mabeau & Fleurence, 1993). Compared to land plants, the chemical composition of seaweeds has been poorly investigated and most of the available information only deals with few species of seaweeds (Burtin, 2003). The chemical composition of seaweeds varies with species, habitat, maturity and environmental conditions. In general, seaweeds are rich in non-starch polysaccharides, minerals and vitamins (Darcy-Vrillon, 1993; Mabeau & Fleurence, 1993). As seaweed polysaccharides cannot be entirely digested by human intestinal enzymes, they are regarded as a new source of dietary fibre and food ingredients (Lahaye, 1991; Mabeau & Fleurence, 1993).

1.6.1. Proximate composition

Seaweeds are a valuable food source as they contain protein, lipids, vitamins and minerals (Burtin, 2003). Seaweeds are not only a useful food source to humans, whole plants and seaweed mixes have been used in animal nutrition (Burtin, 2003) and fish feed (McHugh, 2003). The high vitamin and mineral contents of edible seaweeds make them nutritionally valuable. In addition to vitamins and mineral nutrients, seaweeds are also potentially good sources of proteins, polysaccharides and fibre (Lahaye, 1991; Darcy-Vrillon, 1993). However, very few of the world's available seaweed species are used commercially for specific nutritional purposes. In Asia, people have a long tradition of consuming seaweeds as part of their diet while, in the Western countries, the principal uses of seaweeds are as sources of phycocolloids, thickening and gelling agents for various industrial applications including uses in foods (Abbott, 1996; Darcy-Vrillon, 1993; Mabeau & Fleurence,

1993). Proximate composition of some of the *Gracilaria* species is summarized in Table 1.9.

Table 1.9: Proximate composition (% db) of *Gracilaria* species

Species	Ash	Protein	Fat	IDF*	SDF*
<i>G. coronopifolia</i> ¹	53.4	10.5	2.1	N/A	15.2
<i>G. parvispora</i> ¹	48.1	7.6	2.8	N/A	22.9
<i>G. salicornia</i> ¹	52.9	5.6	2.4	N/A	20.0
<i>G. cervicornis</i> ²	10.5	19.7	0.4	5.6	N/A
<i>G. changii</i> ³	22.7	6.9	3.3	N/A	24.7
<i>G. lemaneiformis</i> ⁴	16.66	20.87	0.87	N/A	N/A

Source: 1 (McDermid & Stuercke, 2003), 2 (Marinho-Soriano & Bourret, 2005), 3 (Norziah & Ching, 2000), 4 (Wen *et al.*, 2006)

*IDF represents insoluble dietary fibres

*SDF represents soluble dietary fibres

1.6.2. Mineral composition

Seaweeds draw all their minerals, macro elements and trace elements from the sea. The mineral fraction of some seaweeds accounts for up to 36% of dry matter. The edible marine seaweeds may be an important source of minerals (Burtin, 2003) as they have some of the trace elements which are lacking or very minor in land vegetables. Despite high contents, the linkage of certain minerals with anionic polysaccharides (alginate, agar or carrageenan) might limit their absorption (Mabeau & Fleurence, 1993). The variation in mineral content is related to factors like seaweed phylum, geographical origin, and seasonal, environmental and physiological variations (Mabeau & Fleurence, 1993; McDermid & Stuercke, 2003). Mineral composition of seaweeds is poorly investigated and has usually been studied as metal absorbents. The mineral composition of some of the seaweed species are summarized in Table 1.10.

Table 1.10: Mineral composition (%db) of seaweed species

Species	N	P	K	Mg	Ca	S
<i>Caulerpa lentillifera</i>	2.39	0.16	0.70	1.65	0.95	1.55
<i>Codium reediae</i>	1.80	0.11	0.77	1.72	0.94	4.35
<i>Codium reediae</i>	1.94	0.12	0.82	1.70	0.92	3.94
<i>Enteromorpha flexuosa</i>	1.27	0.10	1.60	1.17	0.74	3.20
<i>Monostroma oxyspermum</i>	2.58	0.35	3.14	1.36	0.58	6.23
<i>Ulva fasciata</i>	3.62	0.22	2.87	2.19	0.47	5.24
<i>Ulva fasciata</i>	3.74	0.22	3.15	2.94	0.39	5.51
<i>Dictyota acutiloba</i>	2.87	0.16	7.26	1.36	1.03	2.21
<i>Dictyota sandvicensis</i>	2.69	0.13	5.57	0.91	1.81	1.66
<i>Sargassum echinocarpum</i>	1.53	0.14	9.50	1.16	1.31	1.16
<i>Sargassum obtusifolium</i>	1.67	0.14	7.90	0.93	1.50	1.41
<i>Ahnfeltiopsis concinna</i>	1.46	0.10	3.01	0.75	0.44	7.48
<i>Ahnfeltiopsis concinna</i>	1.46	0.11	3.00	0.88	0.49	8.07
<i>Chondrus ocellatus</i>	2.62	0.27	2.26	0.92	0.44	7.16
<i>Eucheuma denticulatum</i>	0.93	0.08	12.40	0.76	0.45	7.25
<i>Gracilaria coronopifolia</i>	3.04	0.38	22.16	0.34	0.18	5.25
<i>Gracilaria parvispora</i>	1.48	0.15	16.00	0.49	0.38	3.99
<i>Gracilaria salicornia</i>	1.12	0.17	17.97	0.51	0.73	3.95
<i>Halymenia formosa</i>	3.29	0.21	4.60	1.25	0.53	5.50
<i>Porphyra vietnamensis</i>	2.47	0.25	3.97	0.78	0.29	2.18

Source: Modified from (McDermid & Stuercke, 2003)

1.6.3. Physiochemical properties

Dietary fibre can be divided into soluble and insoluble fractions. The viscosity of soluble dietary fibre is responsible for slower digestion and absorption of nutrients, and lower levels of blood cholesterol and glucose. In contrast, insoluble dietary fibre is characterized by its ability to increase faecal bulk and decrease intestinal transit time (Rupérez & Saura-Calixto, 2001). Seaweeds contain large amounts of polysaccharides, notably cell wall structural polysaccharides that are extruded by the hydrocolloid industry: alginate from brown seaweeds, carrageenans and agar from red seaweeds. When faced with the human intestinal bacteria, most of these polysaccharides (agars, carrageenans, ulvans and fucoidans), are not digested by humans and therefore can be regarded as dietary fibres (Lahaye, 1991). Water-soluble and water-insoluble fibres have been associated with different physiological effects.

The technological interest and physiological effects of dietary fibre are related to their physicochemical properties, which depend on the chemical structure of the constituent polysaccharides. Fleury and Lahaye (1991) reported that the physicochemical properties of seaweed powder could be assumed to reflect those of the fibre present. In addition, since seaweed proteins are closely related to the cell wall polysaccharides, they may also play a role in the physicochemical properties such as water-holding (Fleury & Lahaye, 1991; Rupérez & Saura-Calixto, 2001). Physicochemical properties of seaweeds have been poorly investigated and there is no literature available on *Gracilaria* species.

1.7. Agar

1.7.1. Definition and description

The name agar originated from the Malay word "Agar-agar", the local name in the Dutch East Indies for *Eucheuma muricatum* (spinosum) (Tseng, 1944) which was exported to China for more than a century. For the sake of simplicity agar-agar was shortened to just agar and is now accepted universally whether in the food and other industries or as culture media.

Agar is defined as a strongly gelling hydrocolloid from marine algae. Agar is a complex mixture of polysaccharides extracted from species of red algae known as agarophytes (Duckworth *et al.*, 1971). Its main structure is chemically characterised by repetitive units of D-galactose and 3-6,anhydro-L-galactose, with few variations

and a low content of sulphate esters (Armisen & Galatas, 2000). Agar is essentially a mixture of neutral polymer agarose, pyruvated agarose and sulphate galactans. The structure of agar consists of alternating β -1,3 and α -1,4 linked D and L galactose residues, respectively (Marinho-Soriano, 2001). The chemical nature of agar varies according to the seaweed source, the environment where the seaweeds grow and on the method of preparation of the agar (Armisen & Galatas, 1987).

1.7.2. Extraction techniques

1.7.2.1. Native agar

Different agar extraction techniques from *Gracilaria* species have been described in literature. The general extraction process is shown in Plate 1.4.

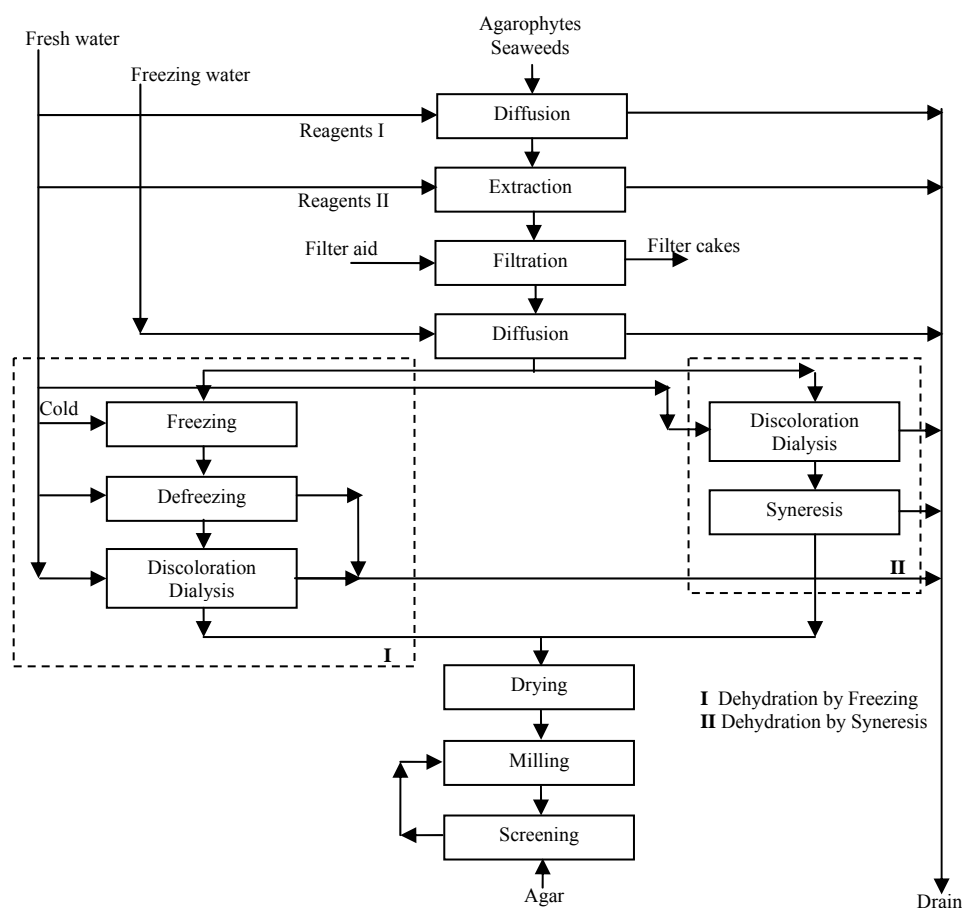


Plate 1.4: Agar extraction process from agarophytes

Source: (Armisen & Galatas, 1987)

In general, agar extraction is done in a waterbath or autoclave and there is no account mentioned of any preference of one over another except Buriyo and Kivaisi

(2003) who reported that agar yield is higher using an autoclave than waterbath. The variables which have commonly been varied in agar extraction without explanation are soaking time, seaweed to water ratio, extraction temperature and time. There are lot of discrepancies in the agar extraction process even for the same species. Extraction variables in the process for different *Gracilaria* species are summarized in Table 1.11 and 1.12.

Table 1.11: Extraction variables for native agar extraction from *Gracilaria* species

SPECIES	Equipment	Soaking Time	Seaweed-water ratio	Extraction	
				Temp. (°C)	Time (h)
<i>G. arcuata</i> ¹	Autoclave	N/A	6g in 200mL and 100mL	120 and 100	1 and 3
<i>G. bursapastoris</i> ²	Autoclave	N/A	10g in 500mL	110	1
<i>G. eucheumoides</i> ³	Autoclave	N/A	20g in 500mL	100 121	1 1
<i>G. vermiculophylla</i> ⁴	Autoclave	N/A	n/a	121	1
<i>G. gracilis</i> ⁴					
<i>G. gracilis</i> ⁵	Waterbath	N/A	40g in 1.2L	boiling	2
<i>G. edulis</i> ⁵					
<i>G. chilensis</i> ⁵					
<i>G. tenuistipitata</i> ⁵	Autoclave	N/A	10g in 500mL	110	1
<i>G. gracilis</i> ⁶					
<i>G. dura</i> ⁶					
<i>G. bursapastoris</i> ⁶					

Source: 1. (Tako *et al.*, 1999) 2. (Marinho-Soriano *et al.*, 1999) 3. (Takano *et al.*, 1995) 4. (Mollet *et al.*, 1998) 5. (Rebello *et al.*, 1997) 6. (Marinho-Soriano, 2001)

Table 1.12: Extraction variables for native agar extraction from *Gracilaria* species continued

SPECIES	Equipment	Soaking Time	Seaweed-water ratio	Extraction	
				Temp. (°C)	Time (h)
<i>G. maramae</i> ⁷	Pressure cooker	N/A	5g in 150mL	120	0.5
<i>G. edulis</i> ⁷					
<i>G. arcuata</i> ⁷					
<i>G. cervicornis</i> ⁸	Waterbath	Over night	10g in 200mL	boiling	1.5
<i>G. blodgettii</i> ⁸					
<i>G. crassissima</i> ⁸					
<i>G. tenuistipitata</i> ⁹	N/A	N/A	20g in 800mL	boiling	1
<i>G. arcuata</i> ⁹					
<i>G. lemaneiformis</i> ¹⁰	Autoclave	N/A	10g in 500mL	115	2
<i>G. dura</i> ¹¹	Autoclave	N/A	10g in 500mL	110	1
<i>G. damaecornis</i> ¹²	Waterbath	N/A	50g in 1.5L	100	3
<i>G. ferox</i> ¹²					
<i>G. domingensis</i> ¹²					
<i>G. compressa</i> ¹²					
<i>G. debilis</i> ¹²					
<i>G. foliifera</i> ¹²	Waterbath	Over night	20g in 500mL	95	3
<i>G. corticata</i> ¹³					
<i>G. crassa</i> ¹³					
<i>G. millardetii</i> ¹³					
<i>G. salicornia</i> ¹³					
<i>G. verrucosa</i> ¹³	Waterbath	Over night	1g in 100 mL	95-100	1
<i>Gracilaria sp.</i> ¹³					
<i>G. cliftonii</i> ¹⁴	Waterbath	Over night	1g in 100 mL	95-100	1

Source: 7. (Falshaw *et al.*, 1999) 8. (Freile-Pelegriñ & Murano, 2005) 9. (Montaño *et al.*, 1999) 10. (Li *et al.*, 2008) 11. (Marinho-Soriano & Bourret, 2005) 12. (Duckworth *et al.*, 1971) 13. (Oyieke, 1993) 14. (Byrne *et al.*, 2002)

1.7.2.2. Alkali treatment of *Gracilaria*

Gracilaria species produce agars with low quality due to its high sulphate concentration. However, the gel properties of many *Gracilaria* agars are reported to be improved by alkali treatment of dry seaweed, which converts L-galactose-6-sulphate in agar to 3,6-anhydro-L-galactose (Duckworth *et al.*, 1971; Freile-Pelegriñ & Murano, 2005; Freile-Pelegriñ & Robledo, 1997b; Orduña-Rojas *et al.*, 2008), which is responsible for the enhancement of the gel forming ability. Alkali treatment of *Gracilaria* including its concentration is reported to be species dependent and variables like temperature and time of treatment must be adjusted to obtain as much desulphation as possible, while still avoiding the yield losses that the alkali treatment process can cause (Armisen & Galatas, 1987; Orduña-Rojas *et al.*, 2008). The alkali treatment conditions and the extraction variables used for agar extraction from different *Gracilaria* species from the most referred literature are summarized in Table 1.13 and Table 1.14 respectively.

Table 1.13: Alkali treatment conditions for different *Gracilaria* species

Species	Equipment	Alkali	Soaking time	Heating	
				Temp. (°C)	Time (h)
<i>G. cornea</i> ¹	Waterbath	0.5, 1, 3, 5 %w/v	ON	80, 85, 90	3
<i>G. canaliculata</i> ²	Waterbath	2g NaOH and 5g CaO	N/A	85-90	3
<i>G. epihippisor</i> ²					
<i>G. abbottiana</i> ²					
<i>G. bursapastoris</i> ²					
<i>G. coronopifolia</i> ²					
<i>G. maramae</i> ³	Waterbath	10g NaOH	N/A	80	3
<i>G. edulis</i> ³					
<i>G. arcuata</i> ³					
<i>G. cervicornis</i> ⁴	Waterbath	3, 5, 7 % w/v	ON	90	3
<i>G. blodgettii</i> ⁴					
<i>G. crassissima</i> ⁴					
<i>G. tenuistipitata</i> ⁵	N/A	10 % (w/w)	N/A	90	2
<i>G. arcuata</i> ⁵					
<i>G. corticata</i> ⁶	Waterbath	2 %w/v	N/A	90	3
<i>G. crassa</i> ⁶					
<i>G. millardetii</i> ⁶					
<i>G. salicornia</i> ⁶					
<i>G. verrucosa</i> ⁶					

Source: 1. (Freile-Pelegrin & Robledo, 1997b), 2. (Santos & Doty, 1983), 3. (Falshaw *et al.*, 1999), 4. (Freile-Pelegrin & Murano, 2005), 5. (Montaño *et al.*, 1999), 6. (Oyieke, 1993)

Table 1.14: Extraction variables after alkali treatment from different *Gracilaria* species

SPECIES	Equipment	Soaking Time	Seaweed - water ratio	Extraction	
				Temp. (°C)	Time (h)
<i>G. cornea</i> ¹	Waterbath	N/A	20g in 600 mL	Boiling	1.3
<i>G. canaliculata</i> ²	Waterbath	N/A	60g in 700 mL	95-100	1
<i>G. epihippisor</i> ²					
<i>G. abbottiana</i> ²					
<i>G. bursapastoris</i> ²					
<i>G. coronopifolia</i> ²					
<i>G. maramae</i> ³	Pressure cooker	ON	5g in 200 mL	120	0.5
<i>G. edulis</i> ³					
<i>G. arcuata</i> ³					
<i>G. cervicornis</i> ⁴	Waterbath	N/A	10g in 300 mL	Boiling	1.5
<i>G. blodgettii</i> ⁴					
<i>G. crassissima</i> ⁴					
<i>G. tenuistipitata</i> ⁵	N/A	N/A	20g in 800 mL	Boiling	1
<i>G. arcuata</i> ⁵					
<i>G. corticata</i> ⁶	Waterbath	ON	20g in 500 mL	95	3
<i>G. crassa</i> ⁶					
<i>G. millardetii</i> ⁶					
<i>G. salicornia</i> ⁶					
<i>G. verrucosa</i> ⁶					

Source: 1. (Freile-Pelegrin & Robledo, 1997b), 2. (Santos & Doty, 1983), 3. (Falshaw *et al.*, 1999), 4. (Freile-Pelegrin & Murano, 2005), 5.(Montaño *et al.*, 1999), 6. (Oyieke, 1993)

1.7.3. Agar properties

Agar quality is determined in terms of its physical (gel strength, gelling temperature and melting point) and chemical properties (sulphate content) which determines its value to industry.

1.7.3.1. Natural population

Native agar yield and properties of *Gracilaria* species from the natural population are summarised in Table 1.15 to 1.17. In general, the reconstitution of agar is in a 1-1.5 % w/v block cured overnight but many authors have not reported the block they used.

Table 1.15: Native agar yield and properties from natural populations of *Gracilaria* species

SPECIES	Agar yield (% db)	Gel strength (g.cm ⁻²)	Melting point (°C)	Gelling temperature (°C)	Sulphate content (°C)
<i>G. arcuata</i> ¹	33.2	N/A	N/A	Room temp.	N/A
<i>G. bursapastoris</i> ²	36-39	22-42 g	n/a	30-36	N/A
<i>G. eucheumoides</i> ³	N/A	N/A	N/A	N/A	N/A
<i>G. vermiculophylla</i> ⁴	17.8	108	N/A	N/A	4.6
	N/A	<20	N/A	N/A	8.8
<i>G. gracilis</i> ⁴	11-19	120-240	N/A	N/A	4.4-6.6
<i>G. gracilis</i> ⁵	N/A	N/A	N/A	N/A	2.9-3.4
					2.4
					2.8
<i>G. edulis</i> ⁵	N/A	N/A	N/A	N/A	2.3

Table 1.16: Native agar yield and properties from natural populations of *Gracilaria* species continued

SPECIES	Agar yield (% db)	Gel strength (g.cm⁻²)	Melting point (°C)	Gelling temperature (°C)	Sulphate content (°C)
<i>G. chilensis</i> ⁵	N/A	N/A	N/A	N/A	2.2
<i>G. tenuistipitata</i> ⁵	N/A	N/A	N/A	N/A	2.3
<i>G. gracilis</i> ⁶	30.0	630	N/A	N/A	1.13
<i>G. dura</i> ⁶	33.5	318	N/A	N/A	1.05
<i>G. bursapastoris</i> ⁶	34.8	22.2	N/A	N/A	1.7
<i>G. maramae</i> ⁷	23	N/A	N/A	N/A	N/A
<i>G. edulis</i> ⁷	37	N/A	N/A	N/A	N/A
<i>G. arcuata</i> ⁷	21	N/A	N/A	N/A	N/A
<i>G. cervicornis</i> ⁸	39.3	<50	54-67	36-37	5.3
<i>G. blodgettii</i> ⁸	~35	<800	86-88	42-45	~3
<i>G. crassissima</i> ⁸	~30	180	~82.5	~40	~4
<i>G. tenuistipitata</i> ⁹	32.9	304	86.5	42.3	3.9 1.18
<i>G. arcuata</i> ⁹	17.2	161	96.2	63.5	4.17 1.17
<i>G. dura</i> ¹⁰	32-35	263-600	N/A	38-43.25	0.97-1.1

Table 1.17: Native agar yield and properties from natural populations of *Gracilaria* species continued

SPECIES	Agar yield (% db)	Gel strength (g.cm⁻²)	Melting point (°C)	Gelling temperature (°C)	Sulphate content (°C)
<i>G. damaecornis</i> ¹¹	N/A	10 g	N/A	N/A	6.0
<i>G. ferox</i> ¹¹	N/A	30 g	N/A	N/A	7.0
<i>G. domingensis</i> ¹¹	N/A	14 g	N/A	N/A	6.0
<i>G. compressa</i> ¹¹	N/A	41 g	N/A	N/A	4.1
<i>G. debilis</i> ¹¹	N/A	140 g	N/A	N/A	3.4
<i>G. foliifera</i> ¹¹	N/A	20 g	N/A	N/A	2.5
<i>G. corticata</i> ¹²	9 -21	<60	82-87.3	29.3-34.9	N/A
<i>G. crassa</i> ¹²	12-25	125-205	91-94.8	36.2-37.2	N/A
<i>G. millardetii</i> ¹²	8-17	60-99	86-92	30.7-34.5	N/A
<i>G. salicornia</i> ¹²	9-15	60-198	85-94	33.6-38.8	N/A
<i>G. verrucosa</i> ¹²	29 -30	205-220	90-91.3	28.9-29.5	N/A
<i>Gracilaria sp.</i> ¹²	14-16	199-203	90-91	39.9-40.4	N/A
<i>G. cliftonii</i> ¹³	52	N/A	N/A	N/A	N/A

Source:1. (Tako *et al.*, 1999), 2. (Marinho-Soriano *et al.*, 1999), 3. (Takano *et al.*, 1995), 4. (Mollet *et al.*, 1998), 5. (Rebello *et al.*, 1997), 6. (Marinho-Soriano, 2001), 7. (Falshaw *et al.*, 1999), 8. (Freile-Pelegrin & Murano, 2005), 9. (Montaño *et al.*, 1999), 10. (Marinho-Soriano & Bourret, 2005), 11. (Duckworth *et al.*, 1971), 12. (Oyieke, 1993), 13. (Byrne *et al.*, 2002)

1.7.3.2. Impact of alkali treatment

Agar yield and properties of *Gracilaria* species after alkali treatment are summarised in Tables 1.18 and 1.19. Alkali treatment of seaweed is performed to increase the gel strength but that is dependent on species and extraction variables as seen from these tables.

Table 1.18: Agar properties after alkali treatment of *Gracilaria*

SPECIES	Agar yield (%db)	Gel strength (g.cm⁻²)	Melting point (°C)	Gelling temperature (°C)	Sulphate content (% db)
<i>G. cornea</i> ¹	14.5-17.6	118-155	77-80	33.8-35.3	3.33-4.25
	18.4-19.9	173-752	76-87	34.2-39.5	2.61-3.69
	17.6-22.1	>950	91-94	42.2-42.5	1.54-1.90
	16.6-19.4	>1500	93-96	42.5-43.0	1.53-1.75
<i>G. canaliculata</i> ²	31.07	295	94.5	49.5	4.32
<i>G. epihippisor</i> ²	30.91	168	81.0	48.3	2.55
<i>G. bursapastoris</i> ²	31.94	181	85.0	35.0	9.54
<i>G. coronopifolia</i> ²	47.42	53	58.0	26.0	11.79
<i>G. maramae</i> ³	14	345	87-88.5	44-46	N/A
<i>G. edulis</i> ³	24	315	85-87.5	34.5-37	N/A
<i>G. arcuata</i> ³	20	310	98-100	43-45.5	N/A

Source:1.(Freile-Pelegrin & Robledo, 1997b), 2.(Santos & Doty, 1983), 3.(Falshaw *et al.*, 1999)

Table 1.19: Agar properties after alkali treatment of *Gracilaria* continued

SPECIES	Agar yield (%db)	Gel strength (g.cm⁻²)	Melting point (°C)	Gelling temperature (°C)	Sulphate content (% db)
	~24	<50	54-67	36-37	5-6
<i>G. cervicornis</i> ⁴	~25	<50	54-67	36-37	5-6
	~25	<50	54-67	36-37	5-6
	26.2	500-750	86-88	42-45	~3.5
<i>G. blodgettii</i> ⁴	~22.5	500-750	86-88	42-45	~3.5
	~22.5	500-750	86-88	42-45	~3.5
	~17.5	~1200	~90	~45	1-1.5
<i>G. crassissima</i> ⁴	~15	~1300	~90	~50	1-1.5
	13.1	1390	93	50	1-1.5
					3.34
<i>G. tenuistipitata</i> ⁵	15.7	606	86.7	42.3	0.84
					3.79
<i>G. arcuata</i> ⁵	18.8	278	98.7	60.7	1.00
<i>G. corticata</i> ⁶	6-8.9	<60	N/A	32.6-33.3	2.7
<i>G. crassa</i> ⁶	12.2	155	N/A	36.2	1.9
<i>G. millardetii</i> ⁶	4.5	<60	N/A	33.8	2.0
<i>G. salicornia</i> ⁶	8.5	210	N/A	37.1	1.2

Source: 4.(Freile-Pelegriin & Murano, 2005), 5.(Montaño *et al.*, 1999), 6.(Oyieke, 1993)

1.7.3.3. Other factors

The effects of season and environmental factors on agar yield and properties have also been reported by various authors and are summarised in Table 1.20.

Table 1.20: Agar yield and property variation, due to seasonal and environmental factors for *Gracilaria* species

SPECIES	Agar yield (%db)	Gel strength (g.cm⁻²)	Melting point (°C)	Gelling temperature (°C)	Sulphate content (%db)
<i>G. arcuata</i> ¹	2.9-21.7	17-260	59-81	38-49.5	5.2
<i>G. salicornia</i> ¹	2.9-15.7	29-147	64-77	44-53	4.2
<i>G. blodgettii</i> ¹	0.1-20.7	29-235	52.5-78	42-49	5.3
<i>G. gracilis</i> ²	19-30.5	229-828	N/A	37-48	1.1-1.6
<i>G. bursapastoris</i> ²	23-36	23-168	N/A	34-46	1.7-2.1
<i>G. cervicornis</i> ³	11-20	N/A	N/A	N/A	N/A
<i>G. eucheumoides</i> ⁴	20-29	225-430	75-86	37-42	3.3-4.3
<i>G. multipartita</i> ⁵	25-30	246-511	85-90	35-45	2.4-5
<i>G. salicornia</i> ⁶	13-30.2	118-251	72-94	N/A	0.8-2.8

Source: 1. (Calumpong *et al.*, 1999), 2.(Marinho-Soriano & Bourret, 2003), 3. (Marinho-Soriano *et al.*, 2001) 4.(Villanueva *et al.*, 1999), 5.(Givernaud *et al.*, 1999), 6. (Buriyo & Kivaisi, 2003)

CHAPTER 2: INTRODUCTION AND GENERAL METHODOLOGY

2.1. INTRODUCTION

Gracilaria species are of commercial importance for the phycocolloid industry and are a major source of agar. In Western Australia, five *Gracilaria* species are found along the coastline around Perth including *Gracilaria cliftonii*, *G. blodgettii*, *G. perissana*, *G. canaliculata* and *G. flagelliformis* (Huisman, 2000). Out of these species *G. cliftonii* has been reported to have an exceptionally high agar yield of 52 %db (Byrne *et al.*, 2002).

Commercial cultivation of *Gracilaria* species began in the late 1980's because of the over-exploitation of wild stocks (Alveal *et al.*, 1997). However, presently almost 90 % of the production of *Gracilaria* species is cultivated (McHugh, 2003). Different authors have reported different culture conditions for *Gracilaria* species (Chapter 1). The most important factors which have been identified for the culture of *Gracilaria* species are light, temperature, pH and salinity (Lobban & Harrison, 1994). *Gracilaria* species are reported to be euryhaline showing a wide range of tolerance towards salinity (Lapointe *et al.*, 1984). Several attempts have been made to culture stenohaline (narrow tolerance to salinity) and euryhaline aquatic species in inland saline water (ISW) with mixed outcomes (CSIRO, 2004; Fielder *et al.*, 2001; Prangnell & Fotedar, 2005; Tantulo & Fotedar, 2007). Prangnell and Fotedar (2005) reported detrimental effects on growth and survival of fish and crustaceans when cultured in raw ISW. The use of ISW to culture marine species has been practiced in many countries like USA, India, and Australia, as the ionic composition is similar to ocean water with the exception of low $[K^+]$ in ISW (Prangnell, 2006; Tantulo, 2007). However, there is a natural variation of ions in different ionic profiles of ISW depending on geographical location and season. In Australia, the increasing salinity of inland water sources is caused by the mobilisation of geologically stored salt through rising water tables which is adversely affecting aquatic and terrestrial ecosystems.

Previous reports have mentioned the potential of red marine macroalgae cultivation as an aid to mitigate the cost of land rehabilitation by making profitable use of these saline wastewaters in Australia (Hedt, 2005). For *Gracilaria* species different growth rates have been observed depending on the culture conditions and species (Santos & Doty, 1983). Chemical composition of seaweeds has been poorly

investigated and most of the available information only deals with few species of seaweeds. Agar is a complex mixture of polysaccharides extracted from species of red algae known as agarophytes (Duckworth *et al.*, 1971). Its characteristics (i.e. yield, gel strength, melting temperature, gelling temperature and sulphate content) and its usage in different applications determine its value to the industry (Marinho-Soriano, 2001).

There is only one published article determining the agar yield of *G. cliftonii* but this focuses mainly on its taxonomy and distribution (Byrne *et al.*, 2002). No published information is available on the chemical composition, physicochemical and agar properties of *G. cliftonii*. In addition, no information is available on the growth rates, culture conditions or environment for *G. cliftonii* in either OW or ISW. The outcomes of this research will provide a better understanding of the life cycle of locally available seaweed species. It will also provide an opportunity to utilise the waste/ under-utilised inland saline water lands. In addition, this research will provide the foundation for commercialisation of new locally available product

2.2. AIM

The aim of this research project is to investigate the effect of different ionic profiles of inland saline water on growth and agar characteristics of *Gracilaria cliftonii*.

2.3. SPECIFIC OBJECTIVES

- Determine the chemical composition, physicochemical properties and agar characteristics of *G. cliftonii* life stages from natural populations
- Compare the productivity of *G. cliftonii* and agar characteristics using different nutrient supplementation in ocean and inland saline water
- Investigate the effect of different ionic profiles of inland saline water on the productivity and agar characteristics of *G. cliftonii*.
- Investigate the influence of alkali treatment of different stages of *G. cliftonii* on the agar characteristics and modify the process of alkali treatment of *G. cliftonii* with different process variables to optimise the agar yield and quality
- Modify agar extraction process to optimise agar yield and quality from *G. cliftonii*

2.4. GENERAL METHODOLOGY

2.4.1. Source of water and *G. cliftonii*

Ocean Water and Inland Saline Water were collected from Hillary's, WA (31° 15'S, 115° 45' E) and Wannamal, WA (31°15'S, 116°05'E) (Plate 2.1), respectively. Ionic profile analysis of water from all trials was conducted by "SGS" (Société Générale de Surveillance) Queens Park, WA using inductively coupled plasma (ICP) spectroscopy. *G. cliftonii* samples for all trials were collected around Point Peron, Shoalwater Islands Marine Park (32° 17' S, 115° 42' E), Perth, WA (Plate). *G. cliftonii* fronds were collected from shallow creeks by free diving and transported in containers filled with ocean water to Curtin Aquatic Research Laboratory (CARL). A total of five collections of *G. cliftonii* were made during January, 2006 to 2008. The first four collections were used for culture trials while the last collection was used for agar trials. Sand, mud and epiphytes were removed manually from each collection by rinsing with seawater. Clean *G. cliftonii* fronds were kept under controlled conditions (23 °C, 16- $\mu\text{mol photon m}^{-2} \text{s}^{-1}$ and 35 ppt).



Plate 2.1: Source of inland saline water for the present study

2.4.2. Experimental units

Twenty-five litre plastic containers with a surface area 0.11 m² and fifty-five litre plastic containers with a surface area 0.15 m² were used for culture trials. Each

container in all the trials was provided with aeration through 3 mm airline connected to an airstone. Incident light of $16 \mu\text{mol photon m}^{-2} \text{s}^{-1}$ (1000 lux) was provided by fluorescent tubes (40W) hanging on the top of the plastic containers at a height of 0.8 m from the container bottom. The light intensity was monitored weekly using a lux-o-meter (Dick Smith Q 1367 Lux & FC Light meter, Australia). A diagram of the experimental system is shown in Figure 2.1.

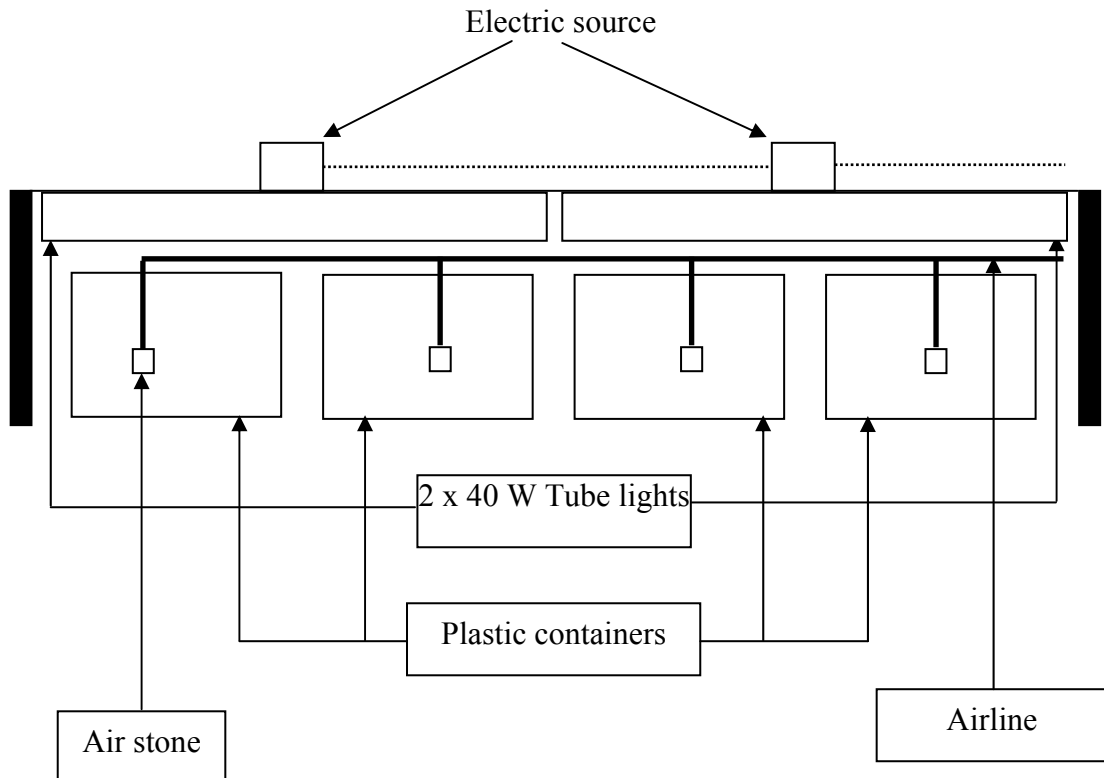


Figure 2.1: Schematic diagram of experimental setup used in culture trials

2.4.3. Growth parameters measurements

The growth parameters of *Gracilaria* species are reported in terms of specific growth rate (SGR) and net yield. There are different ways of calculating SGR (Choi *et al.*, 2006; Dawes, 1995; Haglund & Pedersén, 1993; Marinho-Soriano *et al.*, 2002; Martinez *et al.*, 1990; Nelson *et al.*, 2001; Tetsuro & Young-Meng, 1993; Yang *et al.*, 2005) but the most common method for calculating SGR is described below. Growth can also be determined in terms of apical growth rate which is defined as an exponential increase in the maximum length of a frond and is calculated as described below.

- Apical growth rate is calculated using the formula

$$\text{AGR (\%day}^{-1}\text{)} = ((\text{Ln (L}_f\text{)} - \text{Ln (L}_i\text{)}) / t) \times 100$$

Where, L_f is final length of the frond and L_i is initial length of the frond and t is time in days

- Specific growth rate is calculated using the formula

$$\text{SGR (\%day}^{-1}\text{)} = ((\text{Ln (W}_f\text{)} - \text{Ln (W}_i\text{)}) / t) \times 100$$

Where, W_f is final weight of the frond and W_i is initial weight of the frond and t is time in days

- Net yield is calculated using the formula

$$\text{Net yield (gm}^{-2}\text{day}^{-1}\text{)} = (W_f - W_i) / t / S$$

Where, W_f is final weight of the frond and W_i is initial weight of the frond, t is time in days and S is surface area of container

2.4.4. Chemical Composition

G. cliftonii samples both from natural populations and from culture were freeze-dried in a Heto freeze drier and milled to pass a 1 mm screen using a Christy and Norris cross beater mill prior to determination of proximate and mineral composition. All compositional analysis of protein, ash, nitrogen free extract, crude fibre and mineral composition were conducted according to NATA specifications with proximate and neutral detergent fibre analysis conducted using standard AACC (1995) and AOAC (1995) methods at the Chemistry Centre, East Perth, WA. The macro and trace element content was determined using Inductive Coupled Plasma-Atomic Emission Spectroscopy after acid digestion (McQuaaker *et al.*, 1979) also at the Chemistry Centre, East Perth, WA.

2.4.5. Physicochemical Properties

Physicochemical properties include the water and oil retention capacity of seaweed. Water retention capacity (WRC) and oil retention capacity (ORC) were assessed following the experimental protocol proposed by Jiménez-Escrig and Sánchez-Muniz (2000). To determine WRC, 30 mL of distilled water was added to 500 mg of dry *G. cliftonii* in a 50 mL centrifuge tube. The sample was stirred and left at room temperature for 1 h. After centrifugation at 3000 x g for 20 min, the supernatant was discarded, the residue was weighed and WRC was calculated using the formula

- $WRC\ (g/g) = W_w\ (g) / W_s\ (g)$

Where, W_w is weight of water in g and W_s is weight of dry *G. cliftonii* sample in g

For ORC, the same protocol as mentioned above was followed, using commercial olive oil instead of water. ORC was calculated using the formula

- $ORC\ (g/g) = W_o\ (g) / W_s\ (g)$

Where, W_o is weight of oil retained in g and W_s is weight of dry *G. cliftonii* sample in g

2.4.6. Agar Extraction

G. cliftonii samples were washed with tap water to remove impurities. When the seaweed was free from visible impurities it was then considered as ‘clean seaweed’. The ‘clean seaweed’ sample was oven dried for 8 h at 60 °C and stored in sealed plastic bags for agar extraction. Clean seaweed samples with 3 g dry weight were hydrated in 200 mL of distilled water in 250 mL conical flasks. The conical flasks were covered with aluminium foil to avoid water loss due to evaporation. Agar was extracted from the samples by heating the conical flasks containing samples at 90 °C for 2.5 h in a waterbath. The extracts were filtered using a three ply cheesecloth and were transferred to plastic containers (500 mL). The filtrate was frozen overnight, thawed and oven dried at 60 °C for 24 h and weighed. The quantity of agar was determined in terms of agar yield expressed as percentage dry basis and calculated as

$$\text{Agar Yield (\%db)} = [(\text{Dry weight of agar (g)} / \text{Dry weight of seaweed (g)})] \times 100$$

2.4.7. Agar Properties

The quality of the agar was determined in terms of the physical and chemical properties of the agar: gel strength, melting temperature, gelling temperature and sulphate content. The dried agar was reconstituted in 250 mL glass beakers with distilled water to obtain a 1.5 % w/v agar solution. The hot gel was poured into 50 mL sterile plastic containers and remaining in a glass test tube (2.3 cm diameter, 16.5 cm height) to determine gel strength, melting point and gelling temperature of the agar. These properties were determined after curing gel for 24 h at 25 °C.

Gel strength was determined as described by Marinho-Soriano and Bourret (2005) with minor modifications. A texturometer *TX-2i* with a 1-cm² plunger at a speed of 1 mm s⁻¹ to a depth of 5 mm was used on gel in 50 mL sterile containers. Melting point and gelling temperature were determined using techniques described by Freile-Pelegrin and Murano (2005) with minor modifications. Melting point of the gel in test tubes was measured by placing a glass bead (5 mm diameter) on the gel surface. The test tube rack with test tube was transferred to the water bath at boiling temperature. The melting point was recorded with a digital thermometer when the bead sank into the solution. The same test tubes were kept at room temperature to measure the gelling temperature. The tubes were tilted up and down in a water bath at room temperature until the glass bead ceased moving. The gelling temperature in the tube was immediately measured by introducing a digital thermometer into the agar gel.

Sulphate was determined using the modified AOAC Gravimetric method (AOAC, 1995) with minor modifications. A dry agar sample of 0.5 g was transferred into standard digestion tubes with 10 mL of concentrated HNO₃. The digestion tubes were transferred into a digester set at 123 °C for 30 minutes to have the final volume of digest as 2-3 mL. After cooling the samples in a fume hood, 2-3 drops of 40 % formaldehyde solution were added to reduce the excess HNO₃. The mixture was filtered into a 250 mL conical flask and 0.5 mL concentrated HCl was added followed by distilled water to bring the volume to 200 mL. The solution was heated to boiling and 10 mL of 0.25 M BaCl₂ was added dropwise with constant stirring for 5 minutes and kept aside for 5 h in a warm place. The BaSO₄ precipitate solution was filtered with filter paper (Whatman no. 5) and precipitate was ashed in crucibles in a muffle furnace at 700 °C for 1 h. The crucibles were transferred to a desiccator for cooling and weighed to determine the weight of BaSO₄. The percentage sulphate was calculated as:

$$\text{Sulphate (\%db)} = [(41.16 \times \text{weight of BaSO}_4) / \text{sample weight}] \times 100$$

2.4.8. Statistical Analysis

Statistical software, SPSS v. 16.0 for Windows was used to analyse all data. The data were tested for homogeneity of group variance (Levene's). Growth rates, physicochemical and agar properties were subjected to one-way ANOVA and least

significant difference (LSD) post-hoc test for significant differences at a p-value of less than 0.01 and 0.05. The correlation between growth rates, physicochemical and agar properties were subjected to independent sample t-test at a significance level of $p < 0.05$. Microsoft Excel 2007 was used to determine the correlations and regression equation between two parameters and with respect to the third parameter.

**CHAPTER 3: CHEMICAL PROPERTIES OF *GRACILARIA*
*CLIFTONII***

3.1. INTRODUCTION

Five *Gracilaria* species are found in Western Australia: *Gracilaria cliftonii*, *G. blodgettii*, *G. perissana*, *G. canaliculata* and *G. flagelliformis* (Huisman, 2000). These species are distributed along Western Australian's coastline around Perth, Walkerville in Victoria, and around Tasmania (Withell *et al.*, 1994). *G. cliftonii* is found from Geraldton to Esperance and is high in agar content (52 %db) (Byrne *et al.*, 2002). All Rhodophyta species, including *Gracilaria* are triphasic presenting tetrasporophyte, gametophyte and carposporophyte phases, with alternation between haploid and diploid phases (Lee, 1980).

Chemical composition of seaweeds has been poorly investigated and information is available for few species. The physiological effects of dietary fibre are related to their physicochemical properties, depending on the chemical structure of the constituent polysaccharides (Fleury & Lahaye, 1991) giving seaweeds a major technological interest for food industry applications.

Agar is the main value added product extracted from red seaweeds commonly known as agarophytes (Duckworth *et al.*, 1971). It is a complex mixture of polysaccharides which is chemically characterised by repetitive units of D-galactose and 3-6,anhydro-L-galactose, with few variations and a low content of sulphate esters (Armisen & Galatas, 2000). Agar is essentially a mixture of neutral polymer agarose, pyruvated agarose and sulphate galactans. The structure of agar consists of alternating β -1,3 and α -1,4 linked D and L galactose residues, respectively (Marinho-Soriano, 2001). Its characteristics, yield, gel strength, melting temperature and sulphate content determine its usage in different applications and its value to the industry (Marinho-Soriano, 2001). Gelling temperature, melting point and gel strength are properties commonly used as indicatives of agar quality. These characteristics are closely dependent on the chemical structure of the polysaccharide (Marinho-Soriano *et al.*, 1999).

There is only one published article determining the agar yield of *G. cliftonii* but this focuses mainly on taxonomy and distribution (Byrne *et al.*, 2002). No published information is available on the chemical composition, physicochemical and agar properties for cultivated *G. cliftonii* and culture conditions are not established. These

properties should be determined to ensure reliable results and a basis for research on *G. cliftonii* culture. This chapter provides the chemical composition, physicochemical and agar properties of natural populations of different life stages of *G. cliftonii* and growth rates under ocean and inland saline water culture conditions.

3.2. MATERIAL AND METHODS

3.2.1. Experimental design

G. cliftonii fronds collected from natural populations were washed with tap water, cleaned of impurities and had epiphytes removed. All the fronds were examined under a dissecting microscope and hand sections made to identify the different life stages of *G. cliftonii*. The fronds were separated into carposporophyte, tetrasporophyte, male gametophyte and vegetative. Carposporophyte plants were identified by the presence of protruding cystocarps. Tetrasporophyte plants were identified by the presence of tetrasporangia and male gametophyte plants by spermatangial conceptacles. Thalli without reproductive structures were considered as vegetative. Although male gametophytes were identified the biomass collected was not sufficient to perform any analysis and were thus not shown in results. The specimens were oven dried for 8 h at 60 °C and stored in sealed plastic bags. The samples were analysed for chemical composition, physiochemical properties and agar properties.

3.2.2. Chemical composition

The chemical composition of *G. cliftonii* life stages from natural populations was determined using the methodology described in Chapter 2.

3.2.3. Physicochemical properties

The physicochemical properties of *G. cliftonii* life stages from natural populations were determined using the methodology described in Chapter 2.

3.2.4. Agar extraction and properties

Agar from different life stages of *G. cliftonii* from natural populations was extracted using the methodology described in Chapter 2. Agar yield and properties like gel strength, gelling temperature, melting point and sulphate content were determined by the methodology described in Chapter 2.

3.2.5. Culture

Irrespective of the life stages, *G. cliftonii* fronds were cultured in 55 L plastic containers using three cultivation techniques *viz.* suspended, rope and cage in ocean

water (OW) and inland saline water (ISW) at 35 ppt. In the suspended cultivation technique, fronds were placed at the bottom of a container without any support. In the rope cultivation technique fronds were tied to a nylon rope placed in the middle of the container. In the cage culture technique, fronds were placed in cylindrical plastic cages (length 15 cm and diameter 5 cm) submerged in water and placed in the centre of the container. The experiment was conducted for 10 days in a temperature controlled room (24 ± 1 °C) with a light intensity of $48 \mu\text{mol photon m}^{-2} \text{s}^{-1}$ (3000 lux). No external source of nutrient was provided during the experiment. At the end of the experiment the fronds from each container were weighed using a digital balance and specific growth rate was determined as described in Chapter 2. Due to smaller sample size chemical composition, physicochemical and agar properties from the culture trials were not determined.

3.3. RESULTS

3.3.1. Chemical composition

The proximate composition of *G. cliftonii* life stages from natural populations is shown in Table 3.1. The proximate composition was mainly nitrogen free extract (NFE) which is mainly carbohydrates followed by ash and protein.

Table 3.1: Proximate composition (Mean) (%db) of *G. cliftonii* life stages

Life stage	Protein	Ash	Crude Fibre	NFE
Tetrasporophyte	13.1	36.2	5.1	44.3
Carposporophyte	14.5	33.3	5.2	46.8
Vegetative	11.1	31.2	4.9	51.5

The mineral composition of different life stages of *G. cliftonii* from natural population is shown in Table 3.2 and Table 3.3. The mineral composition of *G. cliftonii* in all the different stages followed the order $\text{K}^+ > \text{Na}^+ > \text{S}^{2+} > \text{N}^{3+} > \text{Mg}^{2+} > \text{Ca}^{2+} > \text{P}^{4+}$.

Table 3.2: Mineral composition (Mean) (%db) of *G. cliftonii* life stages

Life stage	N	P	K	Na	Ca	Mg	S
Tetrasporophyte	2.1	0.14	9.95	5.23	0.60	1.27	2.35
Carposporophyte	2.3	0.14	9.64	2.94	0.37	1.01	2.21
Vegetative	1.8	0.17	7.82	4.62	0.63	1.00	1.55

Table 3.3: Mineral composition (Mean) (mg/kg db) of *G. cliftonii* life stages

Life stage	B	Cu	Fe	Mn	Mo	Zn
Tetrasporophyte	203	4.4	79	78	<.5	89
Carposporophyte	212	5.1	231	116	<.5	74
Vegetative	340	2	120	71	1	210

3.3.2. Physicochemical properties

Water retention capacity (WRC) and oil retention capacity (ORC) of life stages of *G. cliftonii* from natural population is shown in Figure 3.1 and 3.2. WRC of the vegetative stage was significantly higher ($p<0.05$) than the carposporophyte and tetrasporophyte stages while ORC of the vegetative stage was significantly higher ($p<0.05$) than the carposporophyte stage.

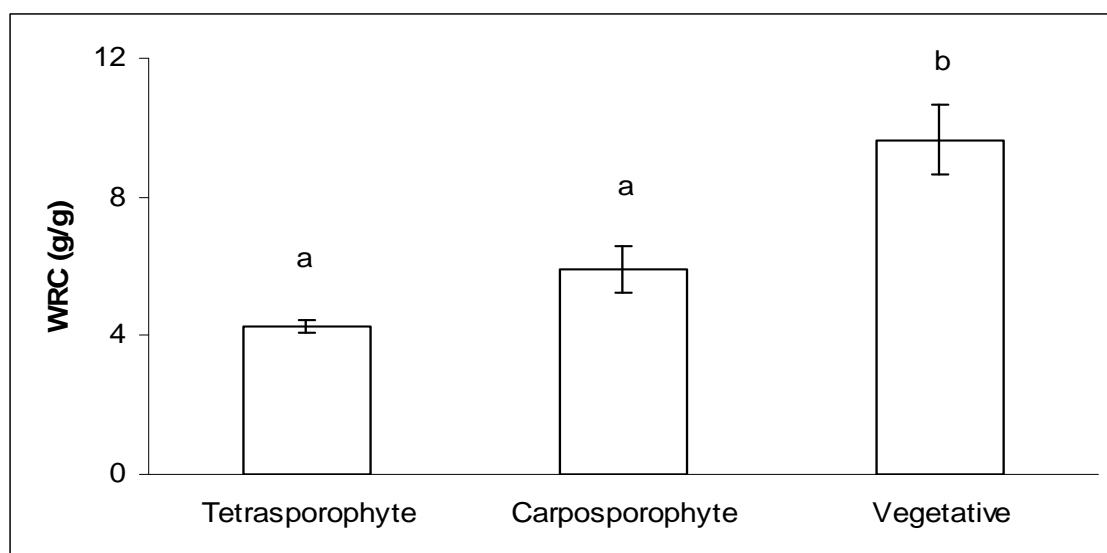


Figure 3.1: Mean water retention capacity (WRC) of *G. cliftonii* life stages.

*Error bars represent standard error of mean. Letters a, b represent the significant differences between life stages

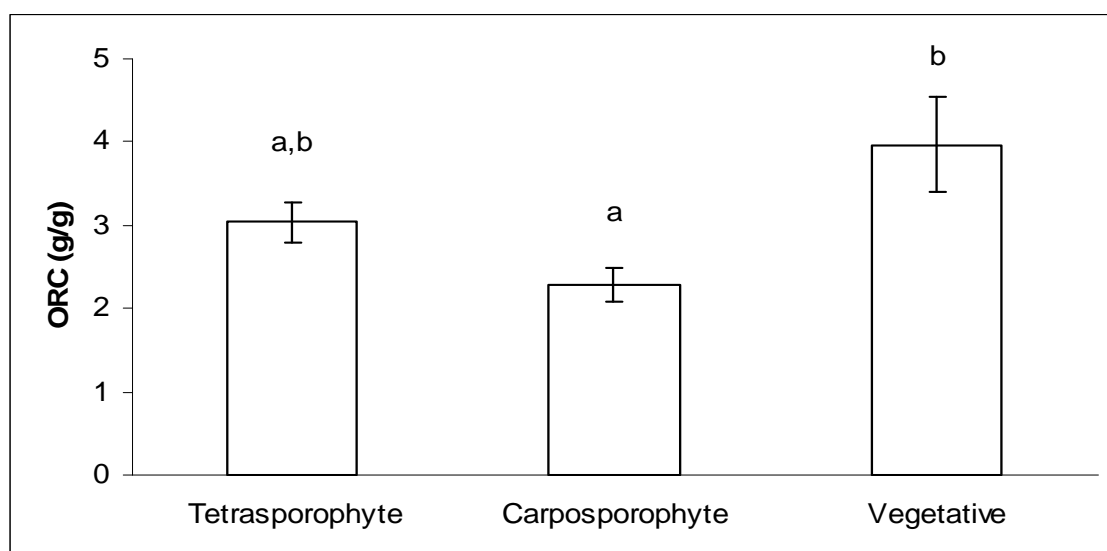


Figure 3.2: Mean oil retention capacity (ORC) of *G. cliftonii* life stages

*Error bars represent standard error of mean. Letters a, b represent the significant differences between life stages

3.3.3. Agar properties

Agar yield and properties of *G. cliftonii* differed among life stage as shown in Table 3.4. Agar yield of carposporophyte and tetrasporophyte stages was significantly higher ($p < 0.05$) than that from the vegetative stage. Agar from different stages of *G. cliftonii* had similar gel strength. However, gelling temperature of agar from the tetrasporophyte stage was significantly lower ($p < 0.05$) than the carposporophyte and vegetative stages. Melting point and sulphate content of agar for all the stages was different to each other.

Table 3.4: Agar yield and properties (Mean \pm S.E.) from life stages of *G. cliftonii*

Life Stage	Agar yield (%db)	Gel strength (g.cm ⁻²)	Gelling temperature (°C)	Melting point (°C)	Sulphate content (%db)
Carposporophyte	56.7 \pm 2 ^a	164 \pm 6 ^a	37 \pm 0.1 ^a	89 \pm 0.0 ^a	6.7 \pm 0.2 ^a
Tetrasporophyte	59.9 \pm 2 ^a	159 \pm 5 ^a	35 \pm 0.5 ^b	87 \pm 0.1 ^b	5.7 \pm 0.2 ^b
Vegetative	49.5 \pm 2 ^b	181 \pm 12 ^a	38 \pm 0.5 ^a	86 \pm 0.6 ^c	7.9 \pm 0.1 ^c

*Letters represent the significant differences between different stages

3.3.4. *G. cliftonii* growth utilising different culture techniques

Specific growth rate (SGR) for *G. cliftonii* under different cultivation techniques is shown in Figure 3.3. SGR of *G. cliftonii* with suspended cultivation in OW was

significantly higher ($p < 0.05$) than cage culture and rope culture in both water types. However, there was no significant difference ($p > 0.05$) in SGR with suspended cultivation in ISW and in OW.

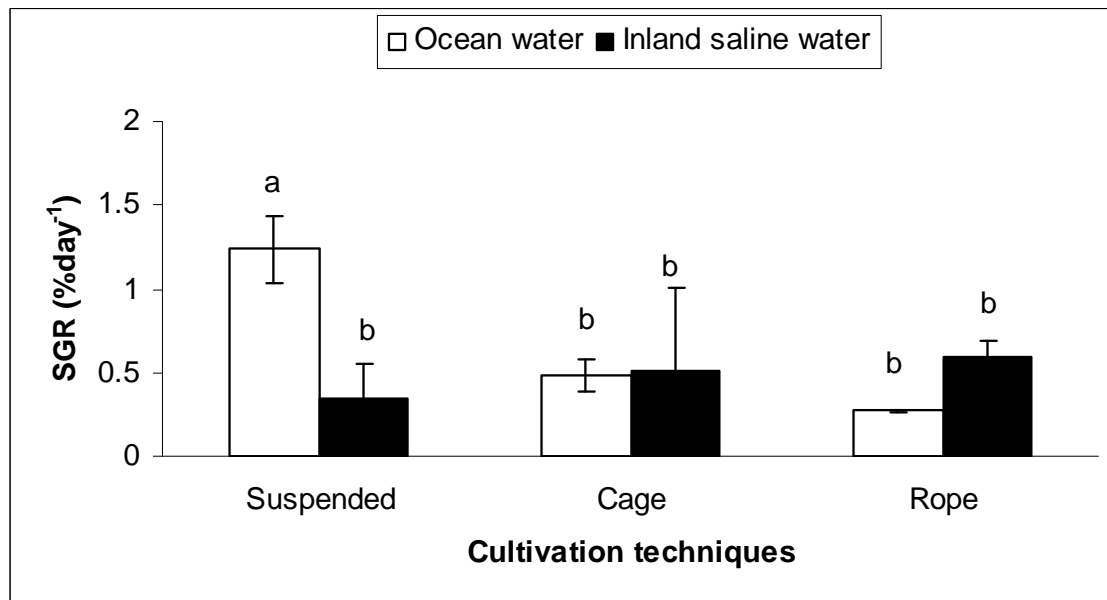


Figure 3.3: Mean specific growth rate (SGR) of *G. cliftonii* with three cultivation techniques in ocean and inland saline water.

*Error bars represent standard error of mean. Letters a, b represent the significant differences between life stages

3.4. DISCUSSION

The present study shows that the chemical composition of *G. cliftonii* is similar to other seaweeds viz. rich in polysaccharides and minerals, followed by protein (Marsham *et al.*, 2007; McDermid & Stuercke, 2003). The carbohydrate and protein content of different stages was higher than that of *G. coronopifolia*, *G. parvispora* and *G. salicornia* (McDermid & Stuercke, 2003). Concentrations of most elements in different stages of *G. cliftonii* are similar to those reported for other *Gracilaria* species (Freile-Pelegrin & Robledo, 1997a; McDermid & Stuercke, 2003) with an exception of K and Fe which were lower and Mg and Zn which were higher compared to *G. coronopifolia*, *G. parvispora* and *G. salicornia* (McDermid & Stuercke, 2003)(Chapter 1). The difference in the mineral composition can be due to the species, environmental conditions around the natural population (Rupérez, 2002) and the methodology used to determine the composition (Rao *et al.*, 2007; Wen *et al.*, 2006).

3.4.1. Agar

Yield, gel strength, gelling temperature, melting point and sulphate content of agar showed variation between life stages of *G. cliftonii*. Agar yield of carposporophyte and tetrasporophyte stages of *G. cliftonii* was higher than the reported yield of 52 %db for same species (Byrne *et al.*, 2002). In addition, agar yield from all *G. cliftonii* stages were higher than reported for *G. bursapastoris*, *G. coronopifolia* and *G. verrucosa* life stages (Hoyle, 1978; Marinho-Soriano *et al.*, 1999; Penniman, 1977; Whyte *et al.*, 1981). The high yield found for the tetrasporophyte stages could be related to the structural difference presented in reproductive stages compared to vegetative plants. The present results are in agreement with those of Penniman (1977), Christiaen, *et al.* (1987) and Whyte, *et al.* (1981) who reported high agar yield from reproductive stages compared to the vegetative stage. Different life stages of *G. cliftonii* showed similar gel strength. These results differ from previous studies conducted by Marinho-Soriano, *et al.* (1999), Penniman (1977), Hoyle (1978) and Whyte *et al.* (1981) who found differences in gel strength from different life stages and that the gel strength of the agar from carposporophyte stages was weaker than that from the vegetative stage. In this study agar from the carposporophyte and vegetative stages showed gelling temperatures higher than that from the tetrasporophyte stage. Marinho-Soriano, *et al.*, (1999) and Whyte, *et al.* (1981) studied the gelling temperature in the life stages of *G. bursapastoris* and *G. gracilis* respectively and showed differences between stages. The present results are in agreement for the carposporophyte stage but contradict for tetrasporophyte and vegetative stages. The variation in gelling temperature of agar reported could be due to the difference in chemical structure of agar that is location of the methoxyl group along with its molecular weight and distribution (Andriamanananatony *et al.*, 2007; Guiseley, 1970; Rebello *et al.*, 1997). Melting point of agar from the vegetative stage was significantly lower compared to reproductive stages. The difference in melting point is also related to the structure of each life stage and agar extraction process. In addition, the difference could be due to the pyruvic acid content of agar (Young *et al.*, 1971). However, the melting point and gelling temperature from different life stages was in the range specified by US Pharmacopeia (82-89 °C and 32-38 °C respectively) for agar usage. The sulphate content of agar from the life stages of *G. cliftonii* are in agreement with studies on *G. bursapastoris* by Marinho-Soriano, *et al.*, (1999) and Whyte *et al.* (1981).

3.4.2. Culture

The highest SGR was observed with suspended cultivation in OW and ISW which demonstrates this as a feasible technique for culturing *G. cliftonii*. Various authors have reported the suspended cultivation of *Gracilaria* under indoor conditions (Buschmann *et al.*, 1996; Buschmann, 2001; Salazar, 1996; Ugarte & Santelices, 1992) with other variable parameters like light, pH and salinity. The present results suggest that *G. cliftonii* can be cultured in ISW but might be a slow growing species. In addition, various parameters such as nutrient requirements, ionic profiles of ISW and ionic ratios formed in these profiles have to be identified.

**CHAPTER 4: EFFECT OF COMMERCIAL NUTRIENT MEDIA ON
GROWTH AND AGAR CHARACTERISTICS IN OCEAN AND
INLAND SALINE WATER**

4.1. INTRODUCTION

The previous trial (Chapter 3) showed that *G. cliftonii* is a slow growing species when cultivated under indoor conditions. This slow growth rate could reflect the low nutrient availability when cultured in OW and ISW without the supply of external nutrients. Nutrient availability is one of the most important operating parameters in the management of seaweed cultivation systems (Lignell & Pedersén, 1987). As a result, many formulations for seaweed nutrition (that is *Gracilaria* sp.) have been elaborated containing different concentrations of macro and micronutrients. These formulations, commonly known as nutrient media, usually include nitrogen and phosphate pulse feeding and are mainly associated with increase in growth rates (apical and specific) and high yields (Capo *et al.*, 1999; Lapointe, 1985; Navarro-Angulo & Robledo, 1999; Smit *et al.*, 1996).

Guillard (1975) and Provasoli (1968) have performed research on assembling numerous formulations of nutrient media for algae culture. Due to the variability in nutrient composition of different media (variable N, P, K and vitamins) most of the research has been limited to a few specific nutrient media like Provasoli's Enriched Seawater (PES) and f2 (Alveal *et al.*, 1997; Friedlander, 1991; Glenn *et al.*, 1999; Martinez *et al.*, 1990; Orduña-Rojas & Robledo, 2002). Alternative to analytical grade inorganic salts, agricultural fertilizers (i.e. urea, animal waste) have been widely used for large scale cultivation but not under laboratory conditions (Asare, 1980b; Gonzalez-Rodriguez & Maestrini, 1984). However, no information is available on the optimum type of nutrient media to be used for *Gracilaria* culture.

The present chapter aims to investigate the effects of different commercially available nutrient media *viz.* f2, Walnes, M-PES and plant fertiliser on growth, physicochemical and agar properties of *G. cliftonii* in ocean and inland saline water.

4.2. MATERIAL AND METHODS

4.2.1. Sample collection

G. cliftonii fronds from the first collection were used and acclimatised for 1 week in controlled conditions as described in Chapter 2.

4.2.2. Commercial Nutrient Media Preparation

Five nutrient media viz. f2, Walnes, two plant fertilisers and Modified-Provasoli's Enriched Seawater (M-PES) were prepared by diluting with distilled water. Walnes and f2 media were prepared using Cell Hi-Walnes P[®] and Cell Hi-f2P[®] (Varicon Aqua Solutions Ltd.). HORTICO Aquasol[™] (Yates, Australia) and Total[™] (Bunnings, Australia) were used as plant fertilisers. M-PES was prepared in laboratory using analytical grade chemicals. The final concentration of micro and macro nutrients, [N], [P], [K], [Trace metal] and [Vitamin] in different commercial nutrient media is shown in Table 4.1.

4.2.3. Experimental design

Two experiments in different volumes of water, 500 mL and 25 L were conducted simultaneously for six weeks each. Both experiments were conducted in ocean water (OW) and inland saline water (ISW) at 35 ppt and the containers without any nutrient media were used as the control. For both experiments strong aeration was provided to maintain water movement in the containers. Full incident light of $16\text{-}\mu\text{mol photon m}^{-2}\text{s}^{-1}$ was provided by fluorescent tubes (40 W) hanging on the top of the containers and was monitored using a lux-o-meter (Dick Smith Q 1367 Lux & FC Light meter). Water was exchanged and fresh nutrient media (20 mL/L) were supplied in the containers weekly.

The first experiment was conducted using four nutrient media (f2, Walnes, Aquasol[™] and Total[™]) with five replicates each in 500 mL containers. The containers were placed in a waterbath maintained at 23 ± 1 °C. The initial inoculum used was one frond of *G. cliftonii* with a 5-cm initial length. Length and fresh weight of fronds from each container were measured every week to determine apical growth rate (AGR) and specific growth rate (SGR) using the formula described in Chapter 2. The final biomass obtained from the first experiment was not sufficient for further agar and physicochemical properties analysis.

The second experiment was conducted in 25 L plastic containers with three nutrient media viz. f2, M-PES and Walnes with four replicates each. Thirty grams of fresh weight of *G. cliftonii* was used as initial biomass in each container. At the end of experiment, fresh weight was measured to determine the SGR and net yield using the formula described in Chapter 2. Experimental setups for both experiments are shown in Figure 4.1 and 4.2.

Table 4.1: Composition of commercial nutrient media used in culturing *G. cliftonii*

	Aquasol™	f2	Walne's	M-PES	Total™
N-P-K source (mg)					
NaNO ₃	-	75	100	35	-
(NH ₂) ₂ CO	18.6	-	-	-	24
KNO ₃	10.4	-	-	-	12
KCl	10.2	-	-	-	-
NaH ₂ PO ₄ .H ₂ O	-	5.65	20	6.5	-
NH ₄ PO ₄	1.8	-	-	-	31
Concentration used (mgL⁻¹)					
Nitrogen	80	75	100	24	35
Phosphorus	3.2	6.5	20	12	6.5
Potassium	14.4	-	-	31	12
N:P:K	25:1:4.5	11:1	5:1	2:1:2.5	5:1:2
Trace metal solution (mg)					
Na ₂ EDTA	0.06	43.6	45	40	-
H ₃ BO ₃	-	-	3.36	5.7	-
Fe(NH ₄) ₂ (SO ₄) ₂ .6H ₂ O	-	-	-	3.51	-
FeCl ₃ .6H ₂ O	-	3.15	1.3	0.49	-
CoCl ₂ .6H ₂ O	-	0.10	0.2	-	-
CoSO ₄ .7H ₂ O	-	-	-	0.024	-
CuSO ₄ .5H ₂ O	0.06	0.10	0.2	-	0.1
MnSO ₄ .H ₂ O	0.15	-	-	0.82	0.26
MnCl ₂ .4H ₂ O	-	0.18	0.36	-	-
Na ₂ MoO ₄ .2H ₂ O	-	0.006	-	-	-
(NH ₄) ₆ Mo ₇ O ₂₄ .4H ₂ O	-	-	0.09	-	-
ZnCl ₂	-	-	0.21	-	-
ZnSO ₄ .7H ₂ O	0.05	0.22	-	1.1	0.1
Vitamin solution (µg)					
Vitamin B ₁₂	-	0.5	100	1.0	-
Thiamine	-	100	100	50	-

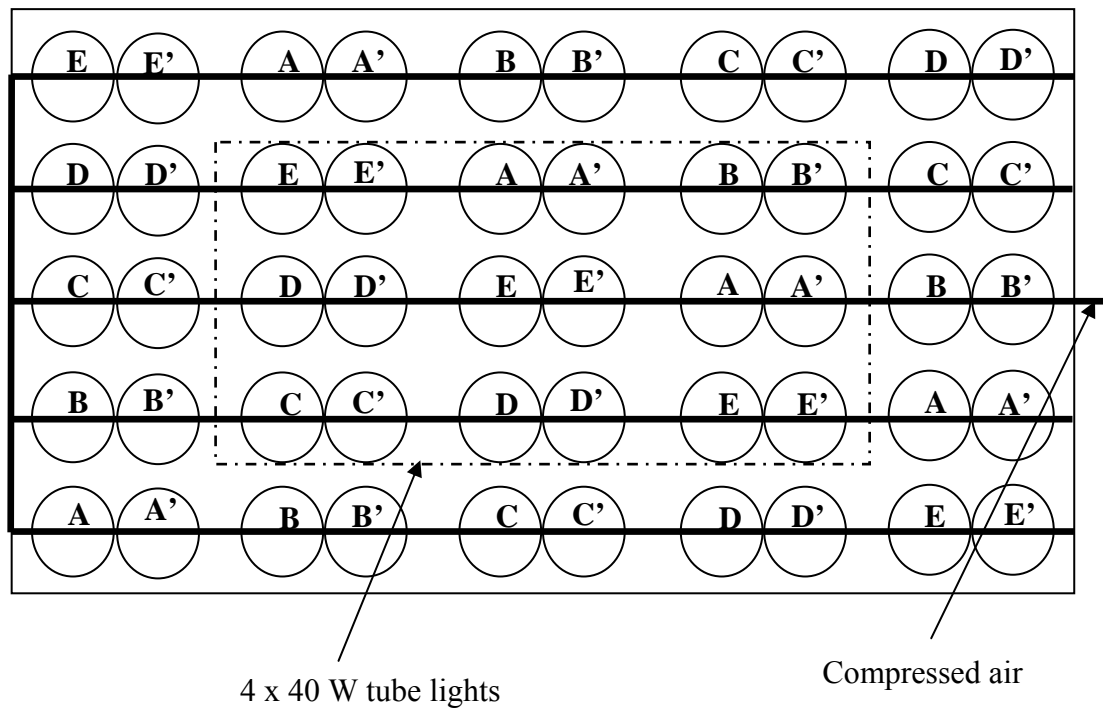


Figure 4.1: Experimental setup for the first experiment in 500 mL containers at 35 ppt without nutrient (A) and with four commercial nutrient media B (Aquasol™), C (f2), D (Walnes), E (Total™) in ocean water and A', B', C', D' and E' represents the same nutrient media in inland saline water

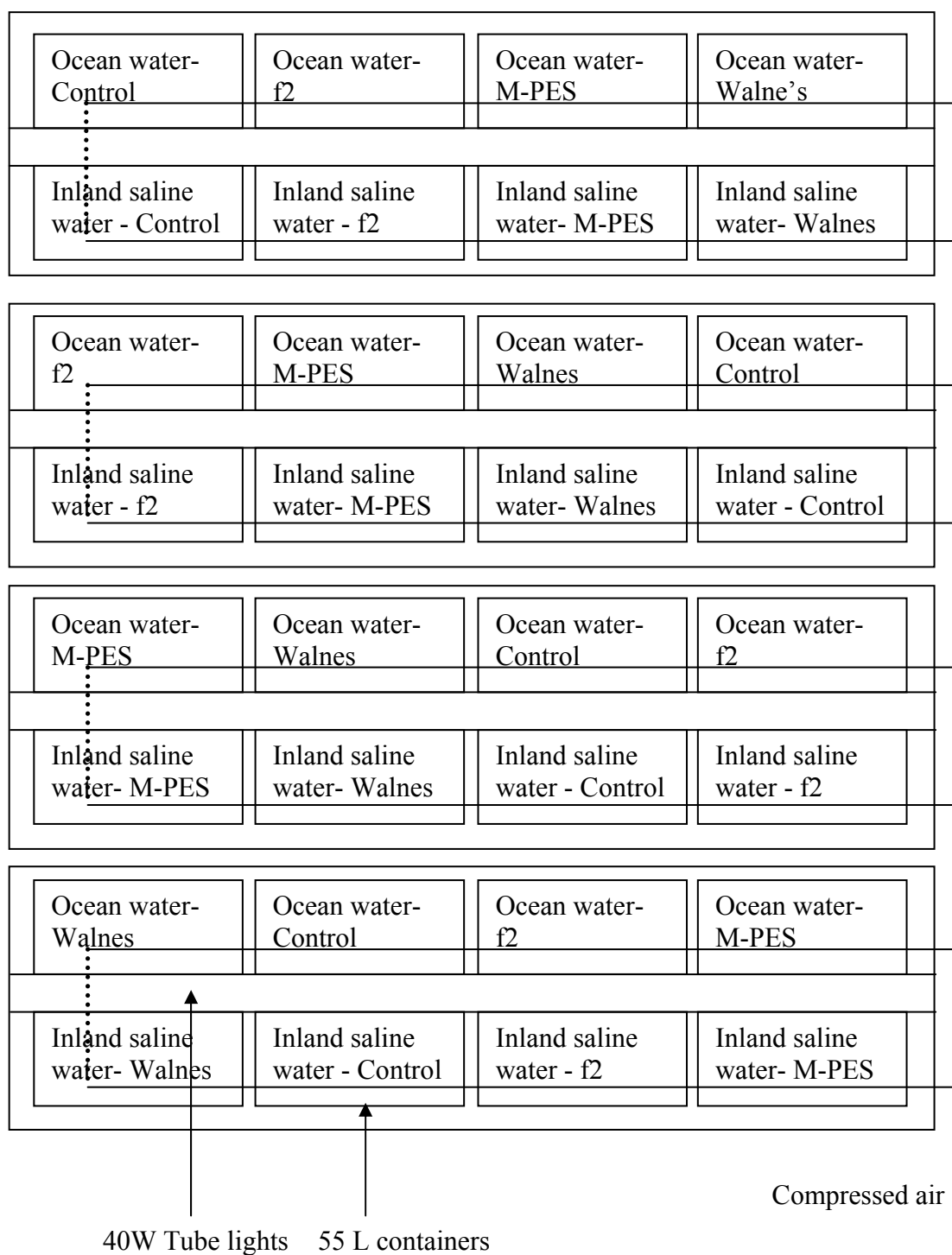


Figure 4.2: Experimental setup for the second experiment in 25 L containers in ocean and inland saline water at 35 ppt with f2, M-PES and Walnes nutrient media.

4.2.4. Physicochemical and agar properties

For the second experiment all samples were oven dried for 8 h at 60 °C and analysed for physicochemical and agar properties. Physicochemical properties (water

and oil retention capacity) and agar characteristics were determined using the methodology described in Chapter 2.

4.3. RESULTS

4.3.1. Growth parameters

SGR of *G. cliftonii* in small volume of water (500 mL) was significantly higher ($p<0.05$) than in large volume of water (25 L) when supplemented with f2 and Walnes nutrient media. The AGR and SGR of *G. cliftonii* from the first experiment are shown in Figure 4.3 and 4.4, respectively. Nutrient media supplementation in OW resulted in significantly higher AGR as compared to control. In addition, irrespective of nutrient media, both growth parameters, AGR and SGR were significantly higher ($p<0.01$) in OW than ISW. Different nutrient media had no influence on AGR in ISW. In f2 and Walnes supplemented OW, SGR was significantly higher ($p<0.05$) than control and AquasolTM.

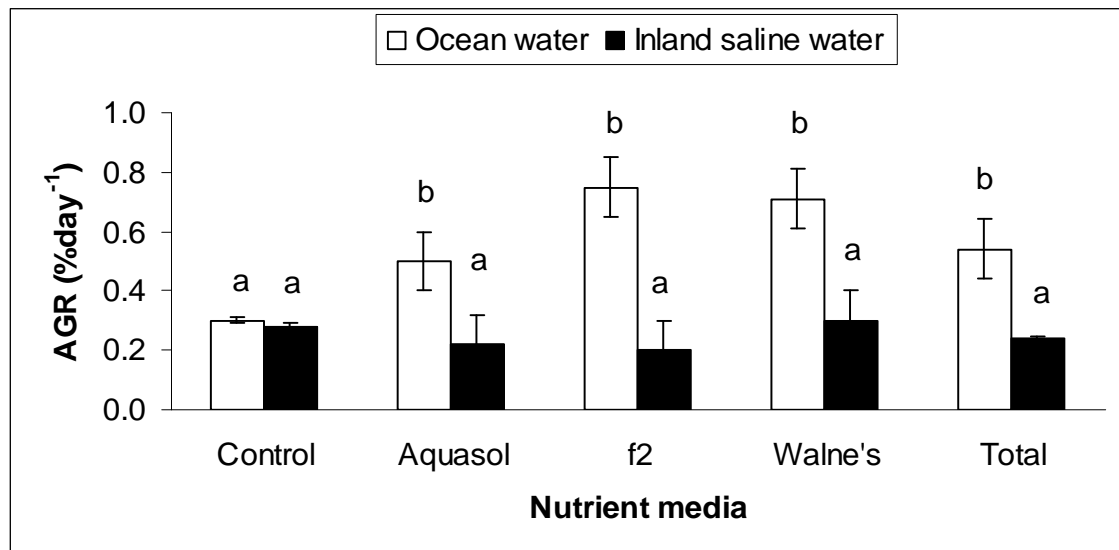


Figure 4.3: Mean apical growth rate (AGR) (%day⁻¹) of *Gracilaria cliftonii* with different nutrient media in ocean and inland saline water.

*Error bars represent standard error of mean. Bars with different letters (a, b, c) are significantly different at a level of $p<0.05$

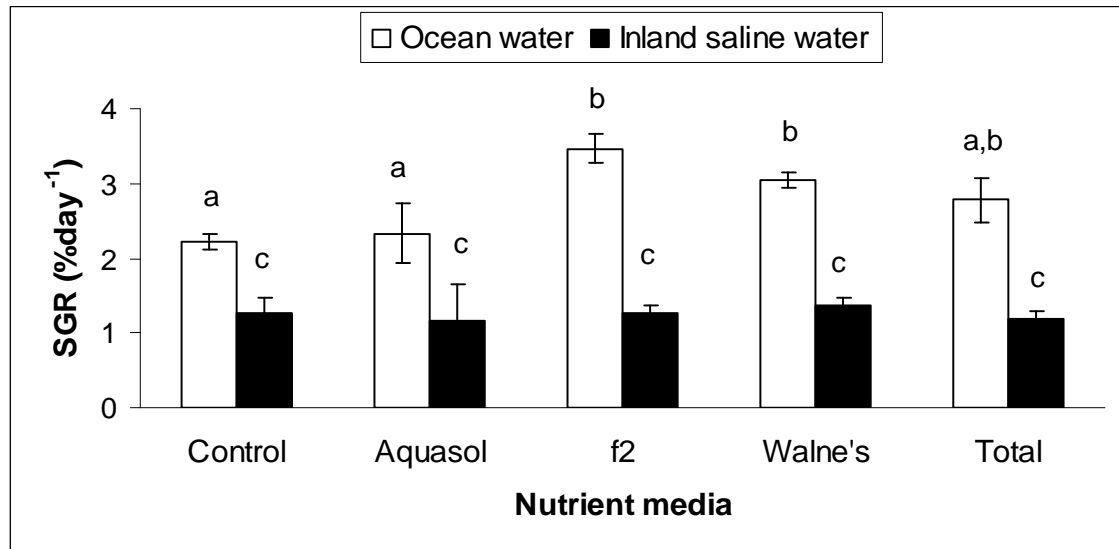


Figure 4.4: Mean specific growth rate (SGR) (%day⁻¹) of *Gracilaria cliftonii* with different nutrient media in ocean and inland saline water

*Error bars represent standard error of mean. Bars with different letters (a, b, c) are significantly different at a level of $p < 0.05$

SGR and net yield of *G. cliftonii* from second experiment are shown in Figure 4.5 and 4.6, respectively. Nutrient media had no influence ($p > 0.05$) on SGR in both water types. However, net yield in Walnes supplemented ISW was significantly higher ($p < 0.05$) than control of ISW and f2 supplemented OW.

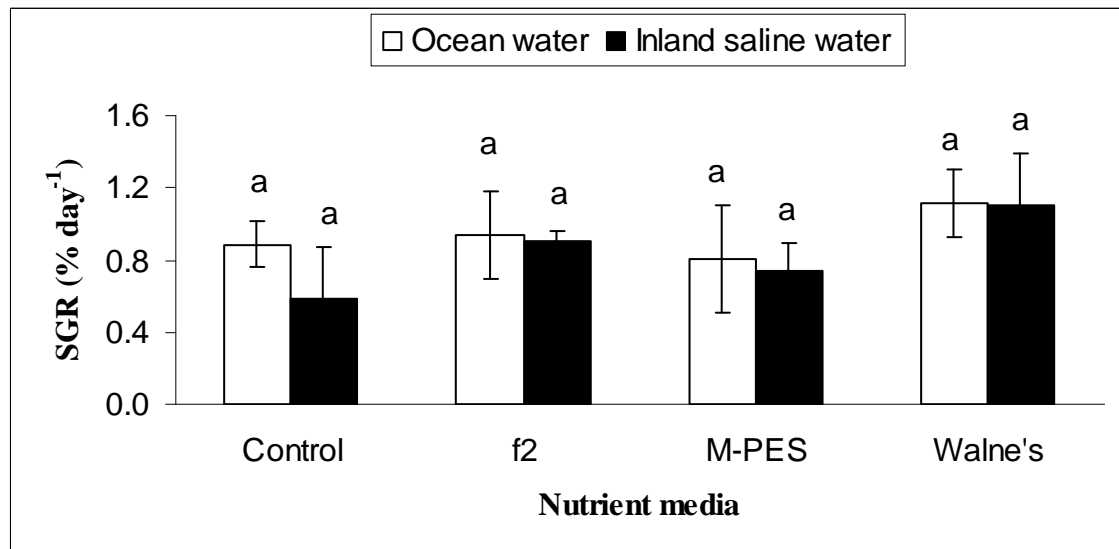


Figure 4.5: Mean specific growth rate (SGR) (%day⁻¹) of *Gracilaria cliftonii* with different nutrient media in ocean and inland saline water

*Error bars represent standard error of mean. Bars with different letters (a, b, c) are significantly different at a level of $p < 0.05$

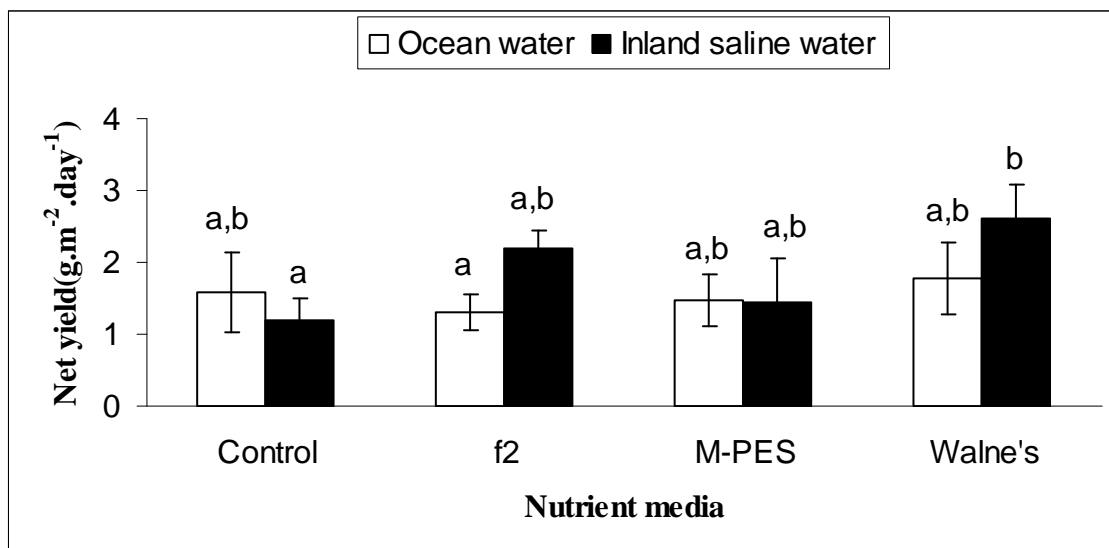


Figure 4.6: Mean net yield ($\text{g.m}^{-2}\text{day}^{-1}$) of *Gracilaria cliftonii* with different nutrient media in ocean and inland saline water

*Error bars represent standard error of mean. Bars with different letters (a, b, c) are significantly different at a level of $p < 0.05$

4.3.2. Physicochemical and agar properties

Variation in water retention capacity (WRC) and oil retention capacity (ORC) of *G. cliftonii* in different commercial nutrient media in both water types are shown in Figure 4.7 A and B. Water retention capacity of *G. cliftonii* in f2 supplemented OW was significantly higher ($p < 0.05$) than control of ISW. Oil retention capacity of *G. cliftonii* in control of ISW and M-PES supplemented ISW was significantly higher ($p < 0.05$) than control of OW and f2 supplemented OW.

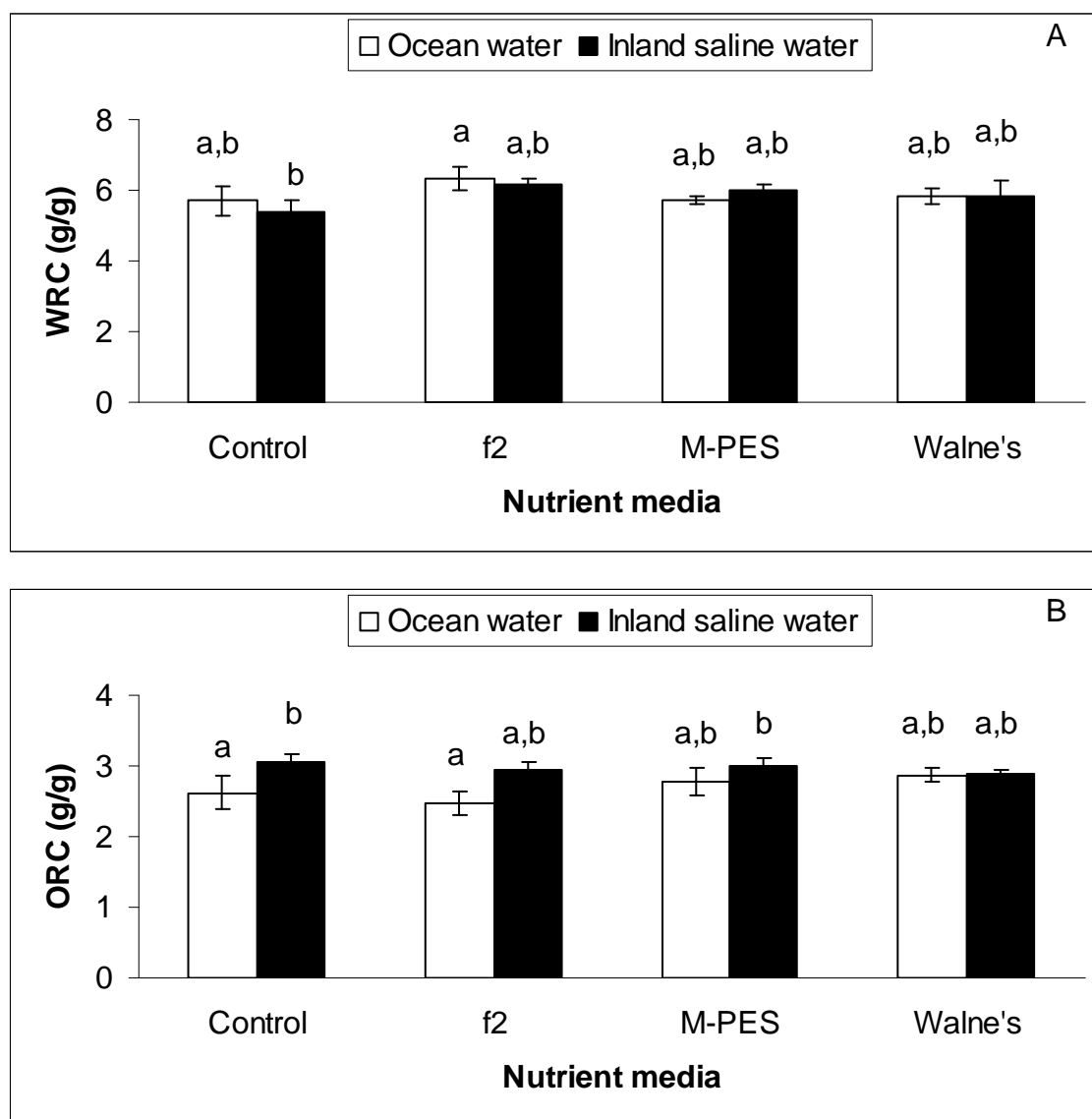


Figure 4.7: (A) Mean water retention capacity (WRC) (g/g) and (B) Mean oil retention capacity (ORC) (g/g) of *Gracilaria cliftonii* with different nutrient media in ocean and inland saline water

*Error bars represent standard error of mean. Bars with different letters (a, b, c) are significantly different at a level of $p < 0.05$

Variations in yield and properties of agar from *G. cliftonii* in culture with different nutrient media in both water types are shown in Table 4.2. Agar yield of *G. cliftonii* in Walnes supplemented OW and f2 supplemented ISW was significantly higher ($p < 0.05$) than M-PES supplemented ISW. Different nutrient media had no influence on agar properties viz. gel strength, melting point, gelling temperature and sulphate content in both water types.

Table 4.2: Agar yield and properties (Mean \pm S.E.) of *Gracilaria cliftonii* cultured with nutrient media in ocean water (OW) and inland saline water (ISW)

Culture conditions	Agar yield (%db)	Gel strength (g.cm ⁻²)	Melting point (°C)	Gelling temperature (°C)	Sulphate content (%db)
OW control	51.8 \pm 4 ^{a,b}	111.8 \pm 3 ^a	84.3 \pm 1.0 ^a	34.5 \pm 0.9 ^a	4.8 \pm 0.9 ^a
OW f2	51.0 \pm 1 ^{a,b}	111.2 \pm 1 ^a	84.7 \pm 1.0 ^a	35.0 \pm 0.8 ^a	5.6 \pm 0.8 ^a
OW M-PES	49.4 \pm 4 ^{a,b}	110.5 \pm 2 ^a	82.8 \pm 0.1 ^a	33.5 \pm 0.3 ^a	5.4 \pm 1.1 ^a
OW Walne's	56.1 \pm 4 ^a	111.0 \pm 1 ^a	83.9 \pm 1.0 ^a	32.9 \pm 0.7 ^a	4.0 \pm 0.2 ^a
ISW control	50.5 \pm 4 ^{a,b}	112.3 \pm 2 ^a	83.6 \pm 0.6 ^a	33.9 \pm 0.8 ^a	5.9 \pm 0.2 ^a
ISW f2	58.3 \pm 5 ^a	115.5 \pm 2 ^a	83.4 \pm 0.5 ^a	34.9 \pm 0.8 ^a	4.9 \pm 0.8 ^a
ISW M-PES	44.0 \pm 4 ^b	116.0 \pm 5 ^a	80.9 \pm 4.0 ^a	34.6 \pm 0.9 ^a	4.7 \pm 0.8 ^a
ISW Walne's	49.7 \pm 1 ^{a,b}	111.9 \pm 2 ^a	85.3 \pm 0.7 ^a	32.9 \pm 0.1 ^a	6.0 \pm 1.1 ^a

*Different letters (a, b, c) are significantly different at a level of $p < 0.05$.

4.4. DISCUSSION

4.4.1. Growth parameters

The SGR of *G. cliftonii* in small volume of water (first experiment) was higher as compared to large volume of water (second experiment) which can be due to the inoculum size/weight and/or surface area to volume ratio (SA:V). In small volume of water, SA: V ratio was higher than the large volume of water resulting in higher nutrient uptake rate and thus higher growth rates. Rosenberg and Ramus (1984) also reported a positive correlation between nutrient uptake rate and SA:V for *Gracilaria tikvahiae* McLachlan. In addition, the final biomass to be obtained from first experiment was not sufficient to investigate the effect of nutrient media on physicochemical and agar properties of *G. cliftonii*.

The results of the present study indicate that the addition and type of nutrient media significantly influence the growth rates of *G. cliftonii* in OW but not in ISW. Numerous studies have examined nitrogen utilisation by *Gracilaria* species in OW and reported as growth-limiting nutrient (Bird *et al.*, 1982; Friedlander, 2001; Hanisak, 1990; Jones *et al.*, 1996; Lignell & Pedersén, 1987; Ryther *et al.*, 1981; Smit, 2002). Therefore, higher SGR observed in f2 and Walnes supplemented OW as compared to control of OW and other media could be due to their high [N] (75 mgL⁻¹ and 100 mgL⁻¹, respectively) in them. However, Aquasol™ has higher [N] (80 mgL⁻¹) as compared to f2 medium (75 mgL⁻¹) but resulted in lower growth rates which could be due to the difference in nitrogen source (NH₄ or NO₃) as shown in *Gracilaria foliifera* (Forsskål) Børgesen (D'Elia & DeBoer, 1978), *G. tikvahiae* (Hanisak, 1990) and *Gracilaria tenuistipitata* Chang and Xia (Haglund & Pedersén, 1993). In Aquasol™, N is supplied from urea and NH₄-N while in f2 media in the form of NO₃-N. The preference for nitrogen source depends on absorption rate which is influenced by the nitrogen status of the seaweed (Hanisak, 1990). However, Navarro-Angulo and Robledo (1999) found no significant differences in *Gracilaria cornea* J. Agardh growth rates with different nitrogen sources.

All nutrient media were ineffective in ISW as low [K⁺] in ISW (90 mgL⁻¹) provides an empowering or overlaying effect (Prangnell, 2006). K⁺ is required for pH control, osmoregulation, protein formation and stability (Lobban & Harrison, 1994) of aquatic species. However, limited studies have been conducted for K⁺ as a limiting nutrient for seaweed growth (Gutknecht, 1965). Gutknecht (1965) reported the absorption of K⁺ by *G. foliifera* as a mechanism to maintain equilibrium between external and internal medium. Plant fertilisers (Aquasol™ and Total™), contain small [K⁺] but do not provide sufficient quantity in ISW compared to OW. In addition, absence of [K⁺] in f2 and Walnes media can deplete nitrogen absorption as N is stored in form of proteins which cannot be formed in the absence of K⁺ (Lobban & Harrison, 1994). The low external [K⁺] can also trigger the osmosis of this ion from tissue to water and thus limiting the growth of *G. cliftonii* in ISW (Gutknecht 1965).

Besides N, P and K seaweeds require a wide variety of inorganic nutrients compared to higher plants, which can quickly become limiting in land-based, low exchange seaweed culture systems (Lobban & Harrison, 1994). In present study, lower SGR with fertilisers (Aquasol™ and Total™) can also be attributed to the

absence of vitamins in these media as compared to f2 and Walnes. However, role of vitamins is not yet established in any macroalgae contrary to microalgae (Lobban & Harrison, 1994).

In the second experiment, higher net yield of *G. cliftonii* in Walnes supplemented ISW as compared to control of ISW and f2 supplemented OW could be due to high [N] of Walnes media. Thus our results confirm the requirement of nitrogen requirement for growth of *G. cliftonii*.

4.4.2. Physicochemical and agar properties

The higher water retention capacity (WRC) in f2 supplemented OW compared to M-PES supplemented ISW could be due to the change in structure of polysaccharides caused by the nutrient supplementations. Roëhrig (1988) reported that physicochemical properties depend on the chemical structure of the constituent polysaccharides which can be altered due to change in external conditions. However, oil retention capacity (ORC) was higher in M-PES-supplemented ISW than f2 supplemented OW which can be due to the hydrophilic nature of the charged polysaccharides of soluble dietary fibres (agar). However, McConnell et al. (1974) reported that the values of ORC are difficult to compare with each other, because they depend on the range of conditions including temperature, time, centrifugation and the preparation of samples.

Gracilaria species cultured under high nitrogen feeding regime and in limiting phosphate concentrations result in higher agar yields as compared to high phosphate concentrations (Lewis & Hanisak, 1996) which is in agreement with the present findings. The difference in the agar yield can be due to high [N] in f2 and Walnes media than M-PES which would have resulted in the alteration of agar structure by the supplementation of different nutrients. The agar isolated from *Gracilaria verrucosa* (Hudson) Papenfuss cultured under nitrogen limited conditions showed large non-polar components (ethanol soluble), minor amounts of methylation and presence of starch, whereas nitrogen supplemented cultures produced more polar agar (hot water soluble) with little starch (Chiles et al., 1989). Bird et al. (1981) and Friedlander (1991) found that increase in nitrogen in culture media result in decreased agar yield but is independent of water type. Low [K⁺] of ISW and/or addition of nutrient media could have altered the structural composition of *G. cliftonii* resulting in difference in agar yield. Thought, the agar yield was

independent of nutrient media and water types but was similar to that from natural populations of *G. cliftonii* (Byrne et al., 2002).

In summary, present study indicates that *G. cliftonii* cultivated with f2, PES and Walne's nutrient media result in similar growth rates but different net yield in OW and ISW. The different N-P-K ratios present in the four nutrient media used indicate could be the most important factor influencing the SGR and should be investigated to define the specific nutrient required for culturing *G. cliftonii*.

**CHAPTER 5: EFFECT OF N-P-K SUPPLEMENTATION ON
GROWTH AND AGAR CHARACTERISTICS IN OCEAN AND
INLAND SALINE WATER**

5.1. INTRODUCTION

In the previous experiments described in Chapter 3 and 4, different nutrient media influenced the growth and agar yield of *G. cliftonii* possibly related to different N-P-K ratios present in these formulations. Different nutrient media provide different composition and concentration of nutrients, however, it is important to find out whether nutrients like N, P and K have an individual effect. Nutrition to seaweed is supplied through nitrogen and phosphate pulse feeding and is associated with increase of growth rates (apical and specific) and high yields (Capo *et al.*, 1999; Friedlander, 1991). For cultivation purposes the development of a specific formulation is required for each species since their nutrient requirements differ (Lignell & Pedersén, 1987).

Nutrient limitation depends on the biogeographical distribution of the targeted species. Tropical and subtropical species i.e. *G. cliftonii* experience low availability of nutrients as tropical waters present low nutrient concentration known as oligotrophic (Hanisak, 1990) conditions observed in Western Australia coastal waters. Different N and P sources and in different ratios has been used in the nutrition of marine algae (Asare, 1980b; D'Elia & DeBoer, 1978; Navarro-Angulo & Robledo, 1999) but not in combination with K. In addition, *Gracilaria* species have higher absorption rate for NH_4 than for NO_3 since NH_4 is directly incorporated into the amino acid pool hence result in higher growth rates (Haglund & Pedersén, 1993; Lobban & Harrison, 1994).

The effect of various nutrients (mainly nitrogen and phosphorus) and seasonal fluctuations on agar characteristics has been previously reported for *Gracilaria* species under indoor and outdoor culture and for natural populations (Buriyo & Kivaisi, 2003; Calumpong *et al.*, 1999; Givernaud *et al.*, 1999; Marinho-Soriano & Bourret, 2003; Marinho-Soriano *et al.*, 2001; Villanueva *et al.*, 1999) but not with different N-P-K ratios. Since nitrogen limitation is known to affect phycocolloid content and growth rate, the nutrient requirements have to be determined, depending on the end use of the seaweed. The present chapter investigates the effect of N-P-K ratios in OW and ISW on the growth, physicochemical and agar properties of *G. cliftonii* in N-P-K supplemented ocean and inland saline water.

5.2. MATERIAL AND METHODS

5.2.1. Sample collection

G. cliftonii fronds from the second collection were used (Chapter 2) and acclimatised for two week under controlled conditions (Chapter 2).

5.2.2. Nutrient Supplementation

Ammonium chloride (analytical grade, Sigma[®], Australia), sodium orthophosphate (analytical grade, Sigma[®], Australia) and potassium chloride (analytical grade, Sigma[®], Australia) salts were used as the source for N, P and K respectively. The salts were dissolved in distilled water to obtain [N] of 75 mgL⁻¹ (equivalent to Guillard f2 medium). Nutrients were supplemented in four different N-P-K ratios *viz.* 1:1:1, 2:1:1, 1:2:1 and 1:1:2. The final concentration of each nutrient in each ratio is shown in Table 5.1.

Table 5.1: Final concentration of N, P, K in different ratios used in for culturing *Gracilaria cliftonii* expressed in mgL⁻¹

N-P-K ratio	[N]	[P]	[K]
1:1:1	75	75	75
2:1:1	150	75	75
1:2:1	75	150	75
1:1:2	75	75	150

5.2.3. Experimental design

The experiment using random block design was conducted for a period of nine weeks in 25 L plastic containers of 2x5x3 (n=30) with two water types (OW and ISW at 35 gL⁻¹), four N-P-K ratios and a control (without supplementation of nutrients). All treatments were used in triplicate. Each container was provided with strong aeration (compressed air through 3 mm airline) for water movement. The experiment was conducted in a temperature controlled room (23 ± 1 °C) with light intensity of 16-μmol.photon.m⁻².s⁻¹. Vegetative fronds of 30 g fresh weight were used as initial biomass and weighed every 3 weeks. Water was changed and fresh nutrients added every 3 weeks to the containers. The specific growth rate and net yield was calculated as described in Chapter 2. All the samples (n=30) were cleaned

with fresh water and oven dried for 8 h at 60 °C. The samples were then analysed for physicochemical properties and agar characteristics as described in Chapter 2.

The experimental setup is shown in Figure 5.1.

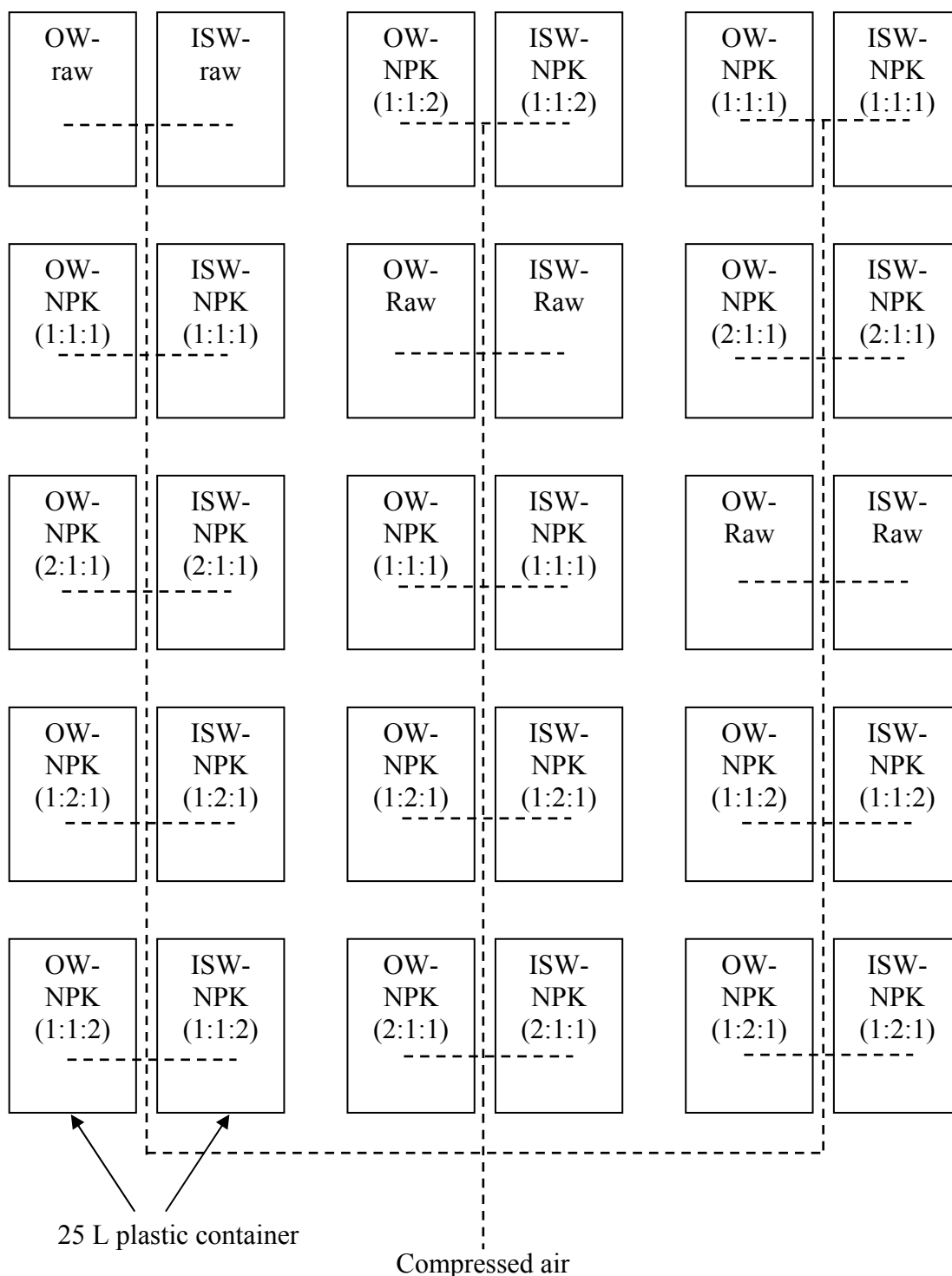


Figure 5.1: Experimental setup for culturing *Gracilaria cliftonii* in N-P-K supplemented ocean water and inland saline water.

5.3. RESULTS

5.3.1. Growth

Specific growth rate (SGR) and net yield of *G. cliftonii* with four N-P-K ratios in OW and ISW are shown in Figure 5.2 and 5.3. SGR and net yield did not change during the entire experiment and showed gradual exponential growth rate with and without nutrient supplementation (Appendix 1). N-P-K supplemented of 1:2:1 in both water types significantly increased the SGR and net yield as compared to other ratios (Figure 1 and 2). However, SGR and net yield with N-P-K supplementation in ratio of 1:2:1 in both water types was similar to control. In addition, net yield with N-P-K supplementation of 1:1:1 was not significantly different to other N-P-K ratios and the control.

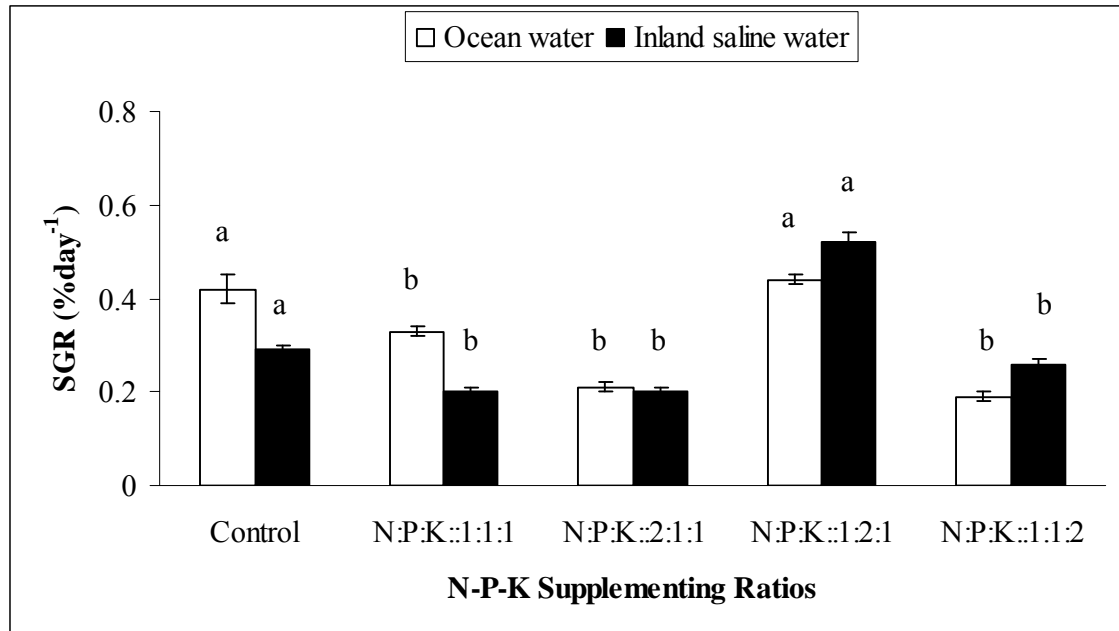


Figure 5.2: Mean specific growth rate (SGR) (%day⁻¹) of *Gracilaria cliftonii* with different N-P-K ratios in ocean and inland saline water

*Error bars represent standard error of mean. Bars with different letters (a, b, c) are significantly different at a level of $p < 0.05$

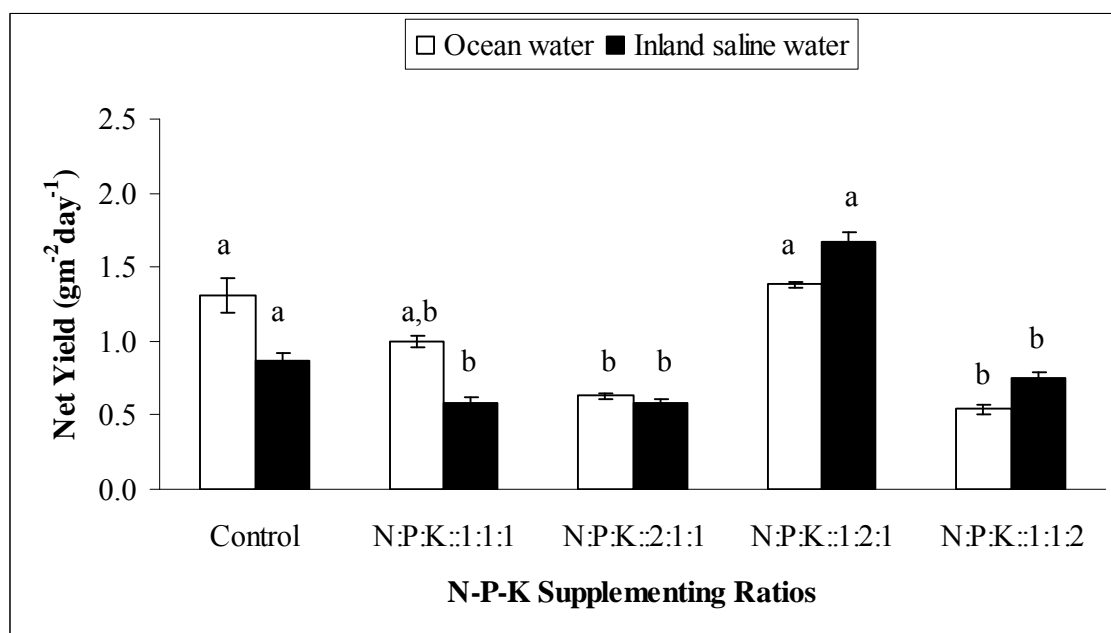


Figure 5.3: Mean net Yield ($\text{g.m}^{-2}\text{day}^{-1}$) of *Gracilaria cliftonii* with different N-P-K ratios in ocean and inland saline water

*Error bars represent standard error of mean. Bars with different letters (a, b, c) are significantly different at a level of $p < 0.05$

5.3.2. Physiochemical properties

The physiochemical properties of *G. cliftonii* with different N-P-K supplementation in both water types are shown in Figure 5.4 A and B. Water retention capacity was in the range $6.4\text{--}8.2 \text{ gg}^{-1}$ but was not influenced ($p > 0.05$) by any nutrient supplementation in OW and ISW. Furthermore, nutrient supplementation had no influence on oil retention capacity (ORC) in OW. However, ORC in N-P-K supplemented ISW in the ratio of 2:1:1 was significantly higher than OW and ISW control, N-P-K supplemented OW, as well as N-P-K supplementation in ratio 1:2:1 in ISW.

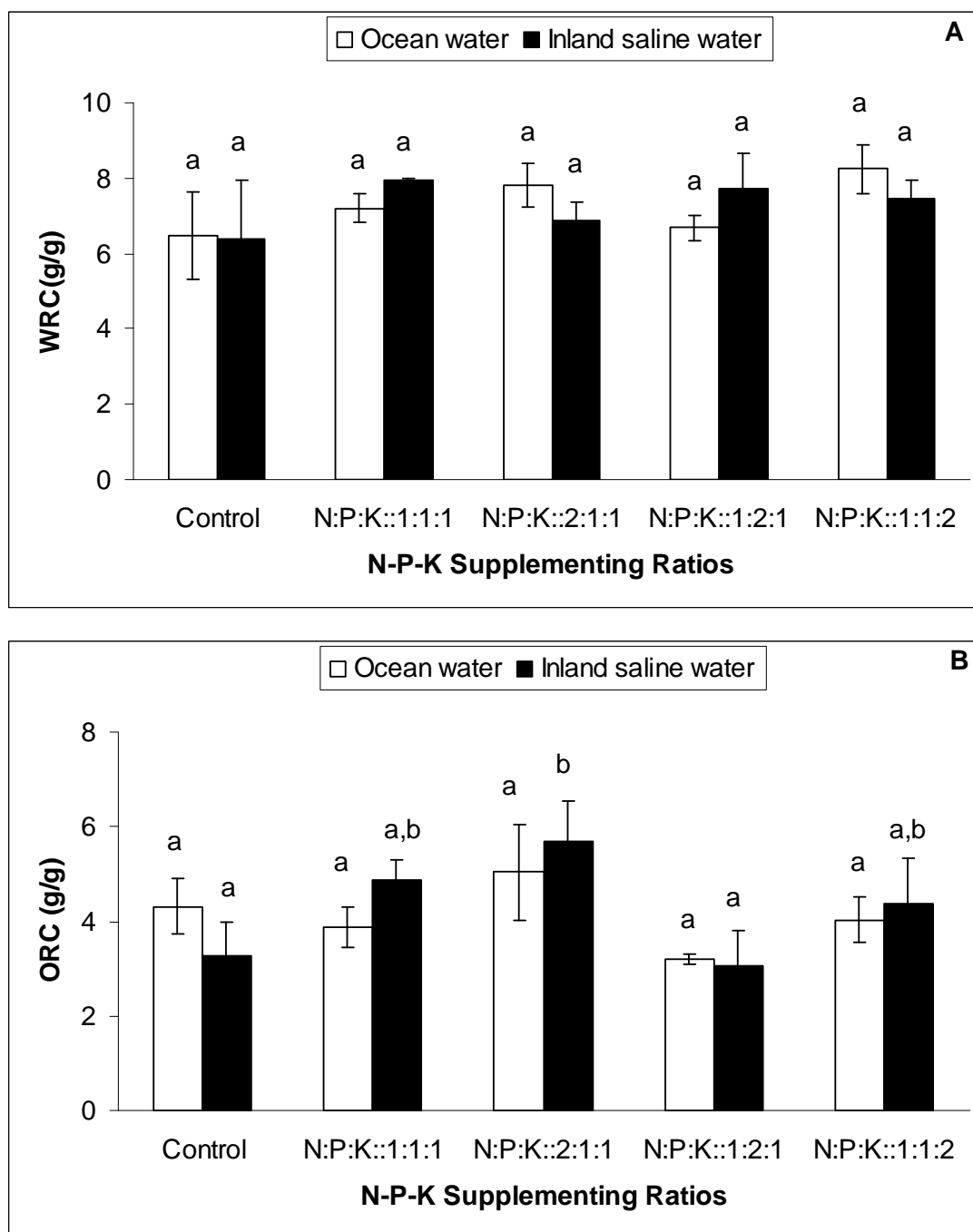


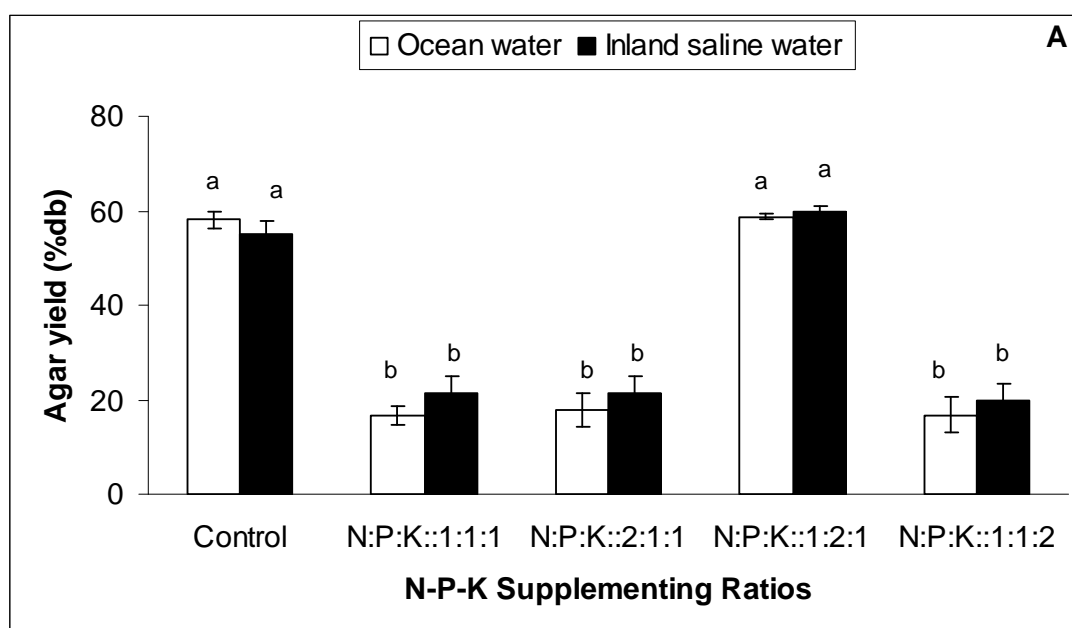
Figure 5.4: (A) Mean water retention capacity (WRC) (g/g) and (B) Mean oil retention capacity (ORC) (g/g) of *Gracilaria cliftonii* cultivated with different N-P-K ratios in ocean and inland saline water

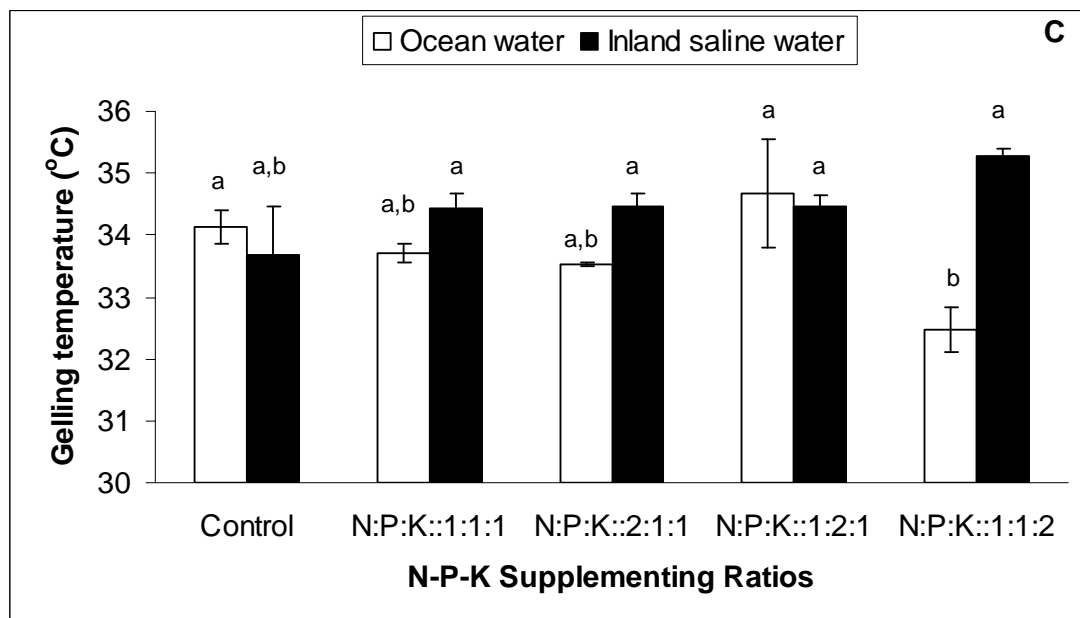
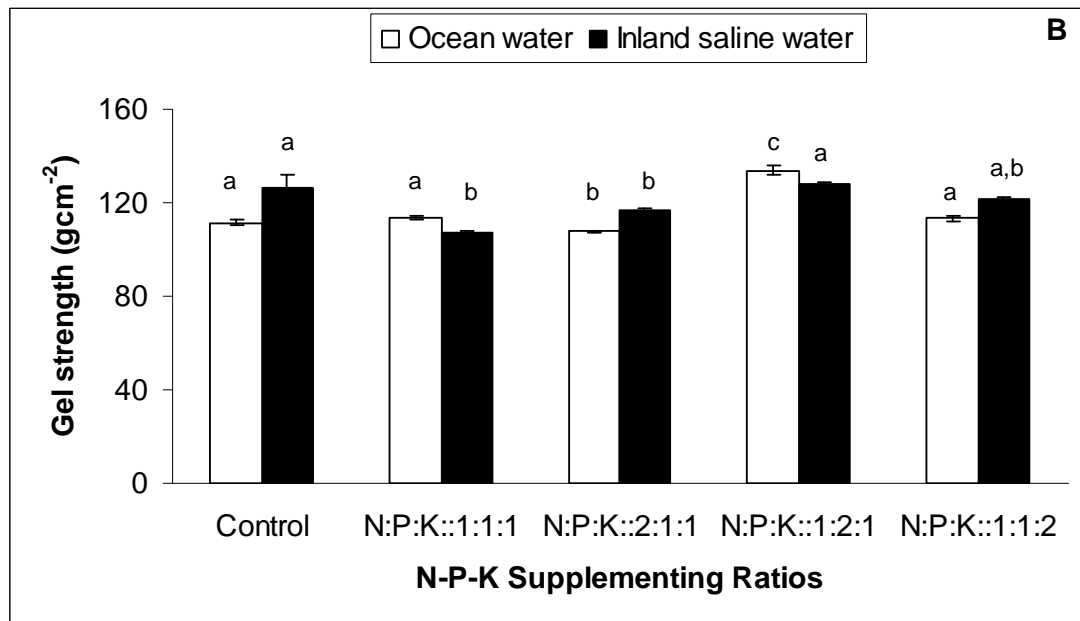
*Error bars represent standard error of mean. Bars with different letters (a, b, c) are significantly different at a level of $p < 0.05$

5.3.3. Agar characteristics

Variation in agar yield and properties with nutrient supplementations in both water types are shown in Figure 5.5 A-E. Agar yield of *G. cliftonii* in OW and ISW

control as well as N-P-K supplementation in ratio of 1:2:1 was significantly higher ($p<0.01$) than other N-P-K ratios in both water types. Gel strength of agar in ISW control, N-P-K supplemented in ratio of 1:2:1 in both water types and N-P-K supplemented ISW in ratio 1:1:2 was significantly higher ($p<0.05$) than OW control and other N-P-K ratios in both water types. In addition, gel strength of agar in N-P-K supplemented OW in ratio of 2:1:1 and ISW in ratio of 1:1:1 was significantly lower ($p<0.05$) than control and other N-P-K ratios. Melting point of agar in OW control and N-P-K supplemented ISW in ratio of 2:1:1 was significantly higher ($p<0.05$) than N-P-K supplemented ISW in ratio of 1:2:1. Gelling temperature of agar in N-P-K supplemented in ratio of 1:1:2 in ISW was significantly higher ($p<0.05$) than in OW. Sulphate content of agar in N-P-K supplemented in ratio of 1:1:1 and 1:1:2 in ISW was significantly higher ($p<0.05$) than control of ISW and N-P-K supplemented OW in ratio of 1:2:1 and 1:1:2.





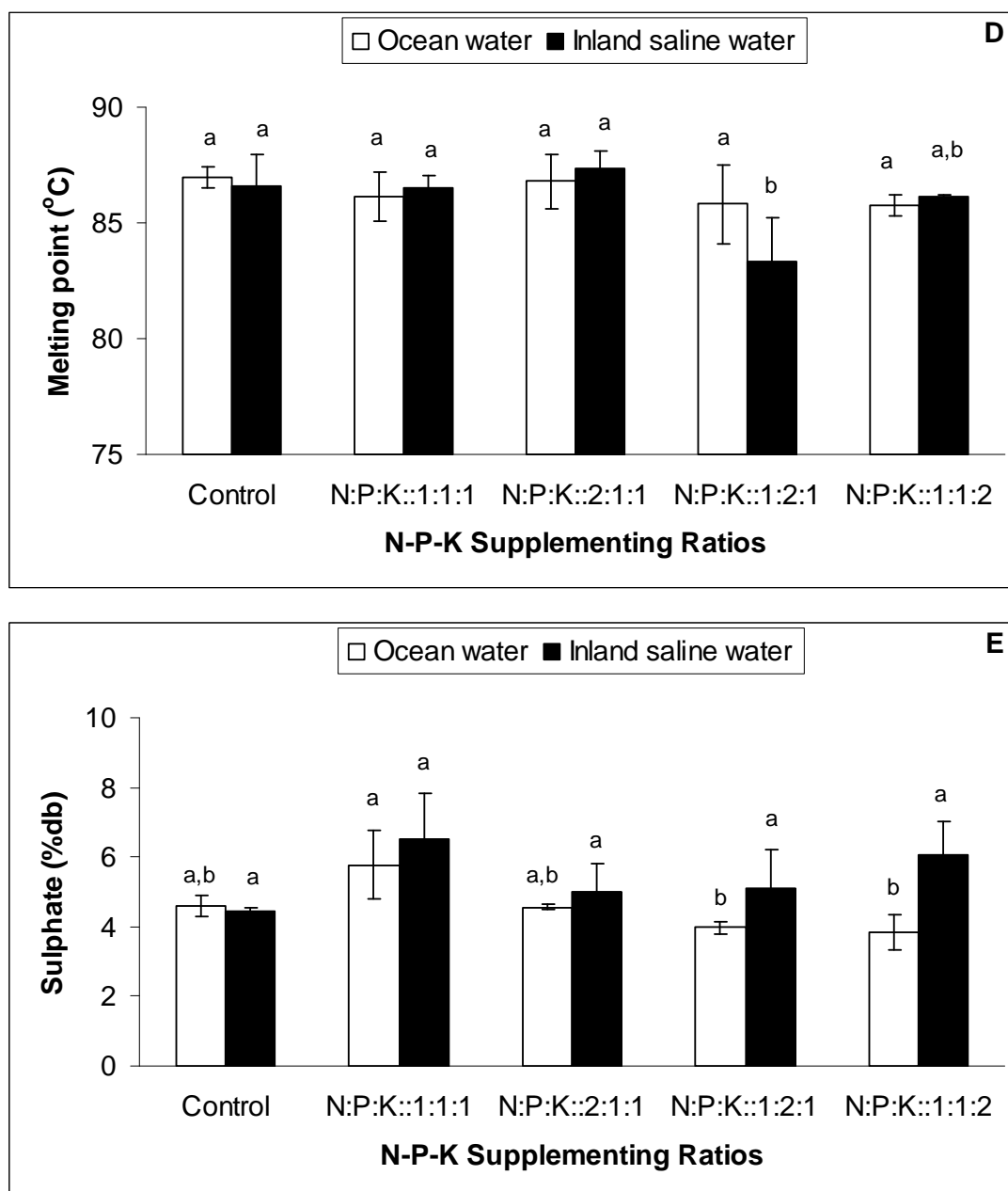


Figure 5.5: (A) Mean yield (% db), (B) Mean gel strength (g.cm⁻²), (C) Mean gelling temperature (°C), (D) Mean melting point (°C) and (E) Mean sulphate content (% db) of agar from *Gracilaria cliftonii* with different N-P-K ratios in ocean and inland saline water

*Error bars represent standard error of mean. Bars with different letters (a, b, c) are significantly different at a level of $p < 0.05$

5.4. DISCUSSION

The present results indicate that higher growth and agar characteristics are obtained when *G. cliftonii* is cultured with supplemented N-P-K in ratio of 1:2:1 independently of the water type (OW or ISW). Nitrogen uptake has been reported for different *Gracilaria* species (Denault *et al.*, 2000; Friedlander, 1991; Lapointe, 1981; Ryther *et al.*, 1981; Smit *et al.*, 1996; Smit, 2002). However, research in phosphorus uptake by *Gracilaria* species is limited and has only been reported in combination with nitrogen (Friedlander, 2001; Navarro-Angulo & Robledo, 1999). In addition, potassium has been identified as contributor in growth and as osmoregulatory and protein stabiliser in seaweeds but has not been studied for *Gracilaria* species. The growth rates of *G. cliftonii* in the present study are lower compared to other *Gracilaria* species (Alveal *et al.*, 1997; Friedlander, 1991; Navarro-Angulo & Robledo, 1999) indicating it as a slow growing species. However growth rates for *G. cliftonii* are not reported under natural conditions so the confirmation would require further studies.

5.4.1. Growth

The high growth rate with nutrient supplementation with N-P-K ratio of 1:2:1 in both water types as compared to other ratio could be due to the similarity in nutrient availability for this species in natural conditions. Lower growth rates with N-P-K ratios of 1:1:1, 2:1:1 and 1:1:2 could be due to the difference in nutrient source used like N and K were provided as chlorides while P was provided as phosphates (Lobban & Harrison, 1994). The high level of chlorides in these ratios would have influenced the growth by affecting pigment concentration and structure (Denault *et al.*, 2000; Lapointe, 1981). The low growth rates in ISW with these ratios can be due to its low [K] in addition to chlorine toxicity. Different nitrogen sources showing different absorption rates have been reported for *G. foliifera* (D'Elia & DeBoer, 1978), *G. tikvahiae* (Hanisak, 1990) and *G. tenuistipitata* (Haglund & Pedersén, 1993), with preference for NH₄-N rather than NO₃-N. However, Navarro-Angulo and Robledo (1999) found no significant differences in *G. cornea* growth rates with different nitrogen sources. Hanisak (1990) reported that the preference for nitrogen source depends on absorption rate which is influenced by the nitrogen status of the seaweed (Hanisak, 1990). As described in Chapter 4, nitrogen supplied in form of NO₃ showed high growth rates with increasing [N]. However, in present experiment lower growth rates were observed with increasing nitrogen content (N-P-

K ratio of 2:1:1), supplied in form of $\text{NH}_4\text{-N}$ indicating that *G. cliftonii* growth could be affected by the nitrogen source.

5.4.2. Physicochemical properties

The physicochemical properties depend on the chemical structure of the constituent polysaccharides (Roëhrig, 1988). However, McConnell et al. (1974) reported that the values of ORC are difficult to compare with each other because they depend on the experimental conditions (temperature, time, centrifugation), as well as on sample preparation. The present study indicates that nutrient supplementation had no influence on water retention capacity (WRC) in both water types. However, high oil retention capacity (ORC) in N-P-K ratio of 2:1:1 in ISW as compared to 1:2:1 can be related to the hydrophilic nature of the charged polysaccharides of soluble dietary fibres (agar). In addition, this might be due to the change in chemical structure of polysaccharides due to the addition and then uptake of different nutrients.

5.4.3. Agar characteristics

In the present study the variations observed on agar characteristics could be due to the structure of agar (Andriamanananatonia et al., 2007; Craigie & Jurgens, 1989; Duckworth & Yaphe, 1971; Lahaye & Rochas, 1991; Lai & Lii, 1997; Murano, 1995), methoxyl groups content (affects gelling temperature) (Guiseley, 1970; Rebello et al., 1997), pyruvic acid content (affect melting point) (Andriamanananatonia et al., 2007; Geresh & Arad, 1991; Lahaye & Rochas, 1991; Young et al., 1971) and sulphate position and content (affects gel strength) (Andriamanananatonia et al., 2007; Craigie & Jurgens, 1989) which might have been altered due to the addition of nutrients. In addition, Armisen and Galatas (1987) showed that agar characteristics are also dependent on extraction process, species and location from where the seaweed was harvested (Chapter 1).

Agar yield of *G. cliftonii* for raw OW, ISW and N-P-K ratio of 1:2:1 (>54 %db) was higher than reported yield for this species (Byrne et al., 2002). In addition, the yield was higher as compared to other cultured *Gracilaria* species (Choi et al., 2006; Hong et al., 2007; Lewis & Hanisak, 1996; Marinho-Soriano & Bourret, 2003; Pickering et al., 1993). The agar yield and gel strength with N-P-K ratio of 1:2:1 in both water types as compared to other ratios is due to the change of structure due to the addition of these nutrients. However, the gel strength was very low as compared to the other cultured *Gracilaria* species (Choi et al., 2006; Hong et al., 2007; Lewis & Hanisak, 1996; Marinho-Soriano & Bourret, 2003; Pickering et al., 1993) but is

reported to increase with alkali treatment (Freile-Pelegrin & Murano, 2005; Freile-Pelegrin & Robledo, 1997b). In addition, high sulphate content of agar from different N-P-K supplemented water types explains the low agar gel strength observed in this study (Armisen & Galatas, 1987). Similarly, the melting point and gelling temperature with different nutrient supplementation in both water types was in range accordingly to United States Pharmacopeia (USP) compendium of quality tests which for gelling temperature is 32-35 °C and melting point is 82-88 °C.

In summary, growth rate, physicochemical and agar properties obtained without nutrient supplementation or with N-P-K ratio 1:2:1 in OW and ISW were not significantly different. The similar growth rate obtained in OW and ISW provides the basis that *G. cliftonii* can be grown in ISW when supplied with proper proportion of nutrients.

**CHAPTER 6: EFFECT OF IONIC PROFILES OF INLAND SALINE
WATER ON GROWTH AND AGAR CHARACTERISTICS**

6.1. INTRODUCTION

In Chapters 4 and 5, it was identified that *G. cliftonii* can be grown in inland saline water (ISW) with different nutrient media and N-P-K supplemented ISW. However, there is a natural variation of ions in ISW depending on geographical locations and season. The use of ISW to culture marine species has been practiced in many countries like USA, India, and Australia, as the ionic composition is similar to ocean water with an exception of low $[K^+]$ in ISW (Prangnell, 2006; Tantulo, 2007). Changes of the internal ionic concentration through salinity and seasonal variations have been reported for *Gracilaria* species (Hurtado-Ponce *et al.*, 1992; Santelices & Doty, 1989; Shang, 1976; Smit *et al.*, 1996; Ugarte & Santelices, 1992) but no published information is available on the effect of change in different ionic profiles of ISW on growth, chemical composition and agar properties of *Gracilaria* species. The ionic profiles of ISW can be altered by lowering the salinity without changing the ionic ratios (Prangnell, 2006; Tantulo, 2007). However, different ionic profiles of ISW can be achieved by mixing certain proportions of OW in ISW (Tantulo, 2007). Mixing ISW with OW resulted in ionic profiles with a significantly lower concentration of major ions like Ca^{2+} , K^+ , Mg^{2+} and Na^+ resulting in different ionic ratios.

This chapter focuses on the effect of different ionic profiles of ISW on the chemical composition, production parameters and agar characteristics of *G. cliftonii*.

6.2. MATERIAL AND METHODS

6.2.1. Sample collection

G. cliftonii fronds from the third collection were used (Chapter 2) and acclimatised for two weeks under controlled conditions (Chapter 2).

6.2.2. Ionic profiles

Eight ionic profiles were achieved by mixing OW, ISW and de-chlorinated fresh water *viz.* $^{35}ISW_0$, $^{35}ISW_{33}$, $^{35}ISW_{66}$, $^{35}ISW_{100}$, $^{25}ISW_0$, $^{25}ISW_{33}$, $^{25}ISW_{66}$ and $^{25}ISW_{100}$. The superscript number represents the salinity of the ionic profile while the subscript number represents the percentage of ISW in the mixture *viz.* the ionic profile $^{35}ISW_0$ represents a mixture of 0 % of ISW and 100 % of OW at 35 ppt,

similarly the ionic profile $^{35}\text{ISW}_{33}$ represents a mixture of 33 % of ISW and 66 % of OW at 35 ppt and so on. An ionic analysis of each ionic profile achieved was conducted by SGS (Société Générale de Surveillance) Queens Park, WA using inductively coupled plasma (ICP) spectroscopy. These particular ionic profiles were selected because it is not clear what inclusion percentage of ISW is required to give comparable growth of *G. cliftonii* as in OW. The concentrations and ratios of major ions of the eight ionic profiles used in the present experiment are shown in Table 6.1.

Table 6.1: Ionic concentrations of major ions (mgL^{-1}) and ionic ratios in different water types at salinities 25 ppt and 35 ppt

Ionic profile	Na⁺	K⁺	Ca²⁺	Mg²⁺	Fe^{2+/3+}	S⁴⁺	Na-K ratio	Ca- Mg ratio
$^{35}\text{ISW}_0$	10752	392	392	1232	0.025	2702	27	0.32
$^{35}\text{ISW}_{33}$	10644	288	470	1470	0.029	2541	37	0.32
$^{35}\text{ISW}_{66}$	10643	189	552	1720	0.033	2406	56	0.32
$^{35}\text{ISW}_{100}$	10750	90	640	1990	0.037	2295	119	0.32
$^{25}\text{ISW}_0$	7680	280	280	880	0.018	1930	27	0.32
$^{25}\text{ISW}_{33}$	7603	206	336	1050	0.021	1815	37	0.32
$^{25}\text{ISW}_{66}$	7602	135	394	1229	0.024	1719	56	0.32
$^{25}\text{ISW}_{100}$	7679	64	457	1421	0.027	1639	120	0.32

6.2.3. Experimental design

To assess the SGR of *G. cliftonii* eight ionic profiles with four replicates were used as an 8 x 4 random block design. The ionic profile $^{35}\text{ISW}_0$ was used as the control. The experiment was conducted in 25 L plastic containers kept in a temperature-controlled room (23 °C) for a period of 4 weeks. Each container was provided with strong aeration for water movement. Full incident light of $16 \mu\text{mol photon m}^{-2} \text{s}^{-1}$ (1000 lux) was provided by fluorescent tubes (40W) hanging on the top of the plastic crates at a height of 0.8 m from the containers bottom. The light intensity was monitored weekly using lux-o-meter (Dick Smith Q 1367 Lux & FC Light meter, Australia). Nutrients were provided twice in the form of 20 mL/L of Provasoli Enriched Seawater (PES) at the end of the second and fourth week. Vegetative fronds of 1.5 g fresh weight of *G. cliftonii* were used as inoculum. The

increase in biomass was registered every week to determine specific growth rate as described in Chapter 2. After four weeks, 20 g fresh weight from each replicate was sampled out from each container and washed with fresh water. 10 g of fresh weight was oven dried at 60 °C for 8 hr and stored in plastic bags for agar extraction and the rest was freeze dried for chemical composition. The growth trial was extended to a total period of six weeks and the biomass left in the containers was analysed for SGR for a further two weeks. The experimental set-up is shown in Figure 6.1

6.2.4. Chemical composition

Chemical composition of samples was determined by the methodology described in Chapter 2. The chemical composition obtained for natural populations of *G. cliftonii* was used to compare with the results obtained in this trial.

6.2.5. Agar extraction

Agar was extracted from natural population and culture samples following the procedure described in Chapter 2. Yield, gel strength, gelling temperature, melting point and sulphate content of extracted agar from all the samples was determined by the methodology described in Chapter 2.

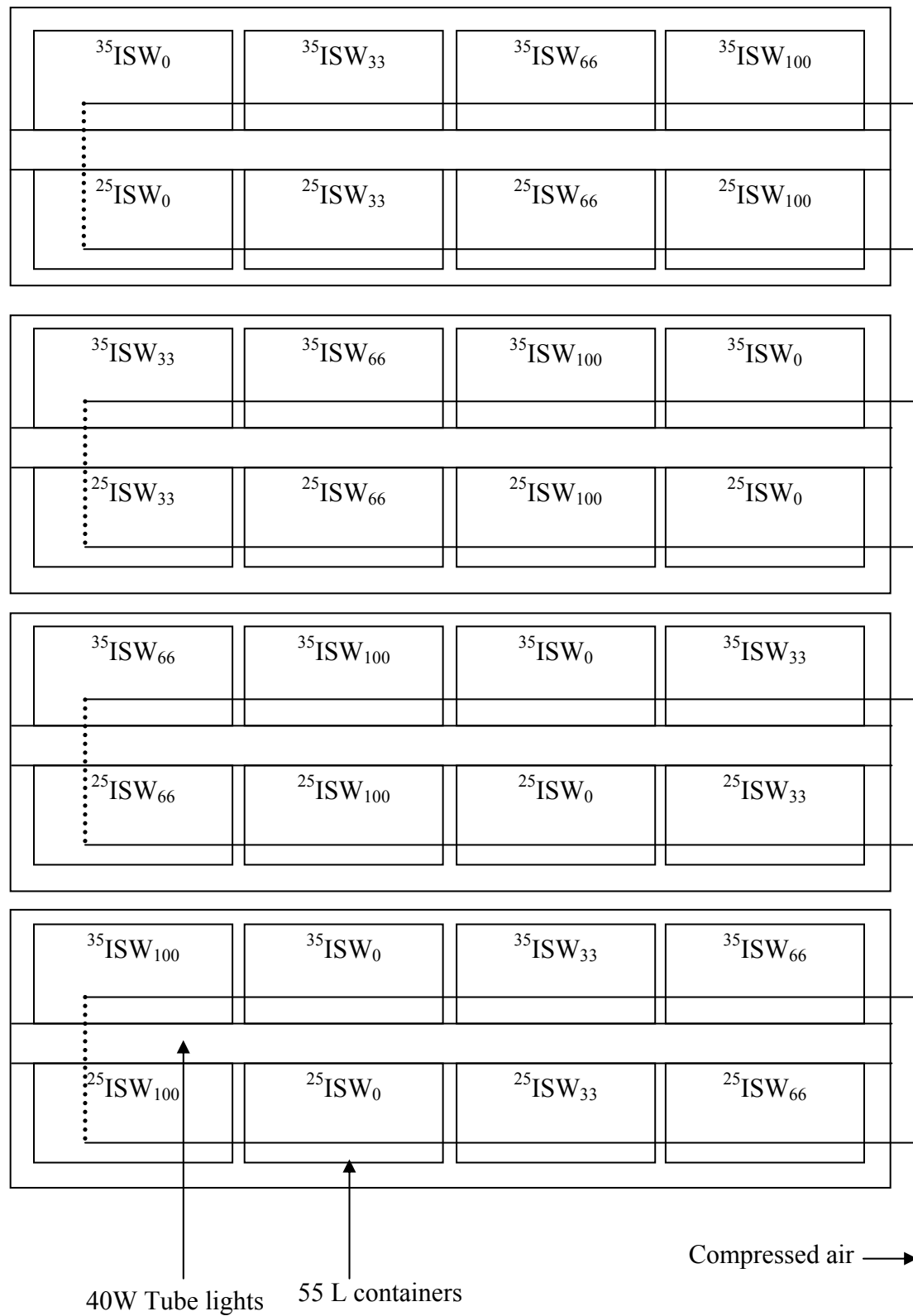


Figure 6.1: Experimental setup for culturing *Gracilaria cliftonii* in different ionic profiles of inland saline water

6.3. RESULTS

6.3.1. Growth

The SGR of *G. cliftonii* varied as a function of salinity and ionic profiles (Table 6.2.) The SGR in the control ($^{35}\text{ISW}_0$) was significantly higher ($p < 0.05$) than in $^{25}\text{ISW}_0$ during the first week. However, during second week different ionic profiles had no influence on SGR. After the third week, SGR in $^{25}\text{ISW}_{33}$ was significantly higher ($p < 0.05$) than SGR in $^{35}\text{ISW}_{33}$. In addition, at the end of the fourth week, SGR in $^{25}\text{ISW}_{33}$ was significantly higher ($p < 0.05$) than in $^{35}\text{ISW}_{33}$. In general the SGR decreased gradually over time.

Table 6.2: Specific growth rate (% day⁻¹) (Mean \pm S.E.) of *Gracilaria cliftonii* in different ionic profiles during four weeks of culture

Ionic profile	WEEK			
	1	2	3	4
$^{35}\text{ISW}_0$	1.60 ± 0.3^a	0.82 ± 0.2^a	$0.52 \pm 0.2^{a,b}$	$0.38 \pm 0.2^{a,b}$
$^{35}\text{ISW}_{33}$	1.45 ± 0.4^a	0.91 ± 0.0^a	0.43 ± 0.1^a	0.16 ± 0.0^a
$^{35}\text{ISW}_{66}$	$1.12 \pm 0.4^{a,b}$	0.84 ± 0.2^a	$0.58 \pm 0.2^{a,b}$	$0.34 \pm 0.2^{a,b}$
$^{35}\text{ISW}_{100}$	1.45 ± 0.2^a	0.81 ± 0.2^a	0.44 ± 0.2^a	$0.27 \pm 0.1^{a,b}$
$^{25}\text{ISW}_0$	0.88 ± 0.4^b	0.88 ± 0.2^a	$0.65 \pm 0.2^{a,b}$	$0.31 \pm 0.1^{a,b}$
$^{25}\text{ISW}_{33}$	$1.25 \pm 0.1^{a,b}$	1.26 ± 0.1^a	0.83 ± 0.1^b	0.51 ± 0.1^b
$^{25}\text{ISW}_{66}$	$1.29 \pm 0.3^{a,b}$	0.93 ± 0.2^a	0.86 ± 0.2^b	$0.24 \pm 0.2^{a,b}$
$^{25}\text{ISW}_{100}$	$1.08 \pm 0.2^{a,b}$	0.97 ± 0.1^a	$0.73 \pm 0.1^{a,b}$	$0.37 \pm 0.0^{a,b}$

Letters as superscript represents significant differences down the column (a, b, c, etc.) (Difference between ionic profiles)

The variation in SGR in different ionic profiles on a weekly basis is shown in Table 6.3. The lowest SGR was observed at the fourth week in all ionic profiles but increased significantly ($p < 0.05$) during the fifth week in all ionic profiles at both salinities. During the sixth week, the highest SGR was observed in $^{25}\text{ISW}_{66}$.

Table 6.3: Specific growth rate (% day⁻¹) (Mean ± S.E.) on individual week by week basis of *Gracilaria cliftonii* in different ionic profiles during 6 weeks of culture

Ionic profile	WEEK					
	1	2	3	4	5	6
³⁵ ISW ₀	1.6 ± 0.6 ^a	0.3 ± 0.0 ^a	0.9 ± 0.1 ^a	0.5 ± 0.2 ^a	2.4 ± 0.6 ^a	1.8 ± 0.5 ^{a,b}
³⁵ ISW ₃₃	1.5 ± 0.9 ^a	0.7 ± 0.3 ^{a,b}	0.4 ± 0.0 ^b	0.4 ± 0.1 ^a	2.1 ± 0.0 ^{a,b}	1.5 ± 0.0 ^a
³⁵ ISW ₆₆	1.1 ± 0.8 ^{a,b}	0.8 ± 0.5 ^b	1.0 ± 0.2 ^a	0.5 ± 0.2 ^a	1.7 ± 0.1 ^b	1.1 ± 0.6 ^a
³⁵ ISW ₁₀₀	1.5 ± 0.4 ^a	0.6 ± 0.2 ^{a,b}	0.8 ± 0.3 ^{a,b}	0.4 ± 0.1 ^a	1.0 ± 0.4 ^{b,c}	1.6 ± 0.6 ^a
²⁵ ISW ₀	0.9 ± 0.9 ^b	0.9 ± 0.3 ^b	0.8 ± 0.1 ^a	0.1 ± 0.0 ^b	2.4 ± 0.2 ^a	1.7 ± 0.3 ^a
²⁵ ISW ₃₃	1.3 ± 0.3 ^{a,b}	1.3 ± 0.2 ^b	0.6 ± 0.2 ^{a,b}	1.0 ± 0.3 ^c	1.8 ± 0.2 ^b	1.4 ± 0.3 ^a
²⁵ ISW ₆₆	1.3 ± 0.7 ^{a,b}	0.6 ± 0.2 ^{a,b}	1.2 ± 0.2 ^a	1.1 ± 0.0 ^c	1.1 ± 0.1 ^{b,c}	2.8 ± 0.8 ^b
²⁵ ISW ₁₀₀	1.1 ± 0.4 ^{a,b}	0.9 ± 0.3 ^b	0.9 ± 0.1 ^a	0.2 ± 0.1 ^{a,b}	0.9 ± 0.4 ^c	1.4 ± 0.6 ^a

Letters as superscript represents significant differences down the column (a, b, c, etc.) (Difference between ionic profiles)

The ions Ca²⁺, Mg²⁺, Fe²⁺, K⁺ and Mg²⁺ showed a strong correlation ($R^2 > 0.9$, $p < 0.05$) between thalli and water at both the salinities. In addition, the ionic ratio of Ca²⁺ to Mg²⁺ also showed a strong correlation ($R^2 > 0.8$, $p < 0.05$) for all ionic profiles irrespective of salinity between thalli and water. However the ionic ratio of monovalent ions (Na⁺/K⁺) was constant and showed no correlation ($R^2 = 0.0$, $p > 0.05$) for all ionic profiles irrespective of salinity between thalli and water.

6.3.2. Chemical composition

The proximate composition of *G. cliftonii* from culture consists mainly of carbohydrates followed by ash and protein. The carbohydrate and ash content of cultured *G. cliftonii* was similar to that observed for the natural population (Table 6.4). The mineral composition of *G. cliftonii* from the natural population has maximum [K⁺] followed by [S] and [Mg²⁺]. [K⁺] and [Mg²⁺] of *G. cliftonii* in culture and natural populations were similar. However, [Ca²⁺] concentration was lower in the control (³⁵ISW₀) compared to the natural population but was similar for other ionic profiles. However, in different ionic profiles at 35 ppt, protein and ash content showed a strong inverse correlation ($R^2 = 0.92$, $p < 0.05$). Similarly carbohydrate and ash content showed an inverse correlation ($R^2 = 0.71$, $p < 0.05$) in different ionic

profiles at 25 ppt. The protein content of *G. cliftonii* in all ionic profiles at both salinities was higher than the natural population. In addition, trace metals that is $[Cu^{2+}]$, $[Fe^{2+}]$ and $[Zn^{2+}]$ were higher in the control as compared to the natural population but were similar in other ionic profiles (Appendix 2).

Table 6.4: Chemical composition (Mean) of *Gracilaria cliftonii* from natural population and in culture in different ionic profiles (% db)

Ionic profile	Protein	CHO	Ash	Na⁺	K⁺	Ca²⁺	Mg²⁺	Fe	S
Natural population	11.1	57.3	31.2	5.2	9.9	0.6	1.3	0.0079	2.4
³⁵ ISW ₀	17.4	53.9	29.8	2.4	10	0.3	1.0	0.0040	2.2
³⁵ ISW ₃₃	18.5	53.7	28.8	2.0	9.9	0.4	1.1	0.0038	2.2
³⁵ ISW ₆₆	19.4	53.0	28.5	2.8	8.1	0.5	1.2	0.0042	1.9
³⁵ ISW ₁₀₀	19.4	54.8	28.0	2.5	9.8	0.5	1.2	0.0043	2.2
²⁵ ISW ₀	17.8	53.0	31.2	2.9	9.8	0.3	1.0	0.0031	2.2
²⁵ ISW ₃₃	19.6	54.1	28.0	2.0	9.9	0.4	0.8	0.0024	1.8
²⁵ ISW ₆₆	18.4	54.7	28.7	2.6	10	0.7	1.0	0.0039	2.1
²⁵ ISW ₁₀₀	19.3	50.2	31.7	2.4	9.8	0.9	1.0	0.0038	2.0

6.3.3. Agar characteristics

The agar yield and properties of cultured *G. cliftonii* and from natural populations are shown in Table 6.5. Agar yield decreased significantly ($p < 0.05$) in all ionic profiles compared to the natural population. In addition, agar yield of *G. cliftonii* in ²⁵ISW₃₃ was significantly lower ($p < 0.05$) than in ³⁵ISW₃₃. However, agar yield of the control (³⁵ISW₀) was not significantly different ($p > 0.05$) to ²⁵ISW₀. In addition, the gel strength of agar in control and ²⁵ISW₆₆ was significantly lower ($p < 0.05$) than in ²⁵ISW₀ and natural population. Different ionic profiles had no influence ($p > 0.05$) on the sulphate content of agar as compared to the natural population. The gelling temperature of agar in control and ²⁵ISW₀ increased significantly ($p < 0.05$) compared to natural population and other ionic profiles. However, agar melting point of the natural population was significantly higher ($p < 0.05$) than the control. In addition, the melting point of agar from ³⁵ISW₆₆ was

significantly higher ($p < 0.05$) than $^{25}\text{ISW}_{66}$. The melting points of agar in $^{35}\text{ISW}_{33}$ and $^{25}\text{ISW}_{66}$ were significantly lower ($p < 0.05$) than other ionic profiles.

Table 6.5: Agar yield and properties (Mean \pm S.E.) of *Gracilaria cliftonii* from the natural population and in culture conditions

Ionic profile	Agar yield (%db)	Gel strength (g.cm²)	Gelling Temperature (° C)	Melting point (° C)	Sulphate content (%db)
Natural population	62 \pm 0.6 ^a	180 \pm 4 ^a	33.2 \pm 0.9 ^a	87.9 \pm 0.4 ^a	5.5 \pm 0.5 ^a
$^{35}\text{ISW}_0$	55 \pm 0.7 ^{b,c}	105 \pm 14 ^b	36.7 \pm 0.2 ^b	83.5 \pm 3.2 ^b	6.5 \pm 0.9 ^a
$^{35}\text{ISW}_{33}$	57 \pm 1.4 ^b	149 \pm 10 ^{a,b}	34.9 \pm 0.7 ^{a,b}	80.1 \pm 3.8 ^b	5.0 \pm 0.7 ^a
$^{35}\text{ISW}_{66}$	55 \pm 2.1 ^{b,c}	157 \pm 7 ^{a,b}	34.8 \pm 0.3 ^a	87.6 \pm 0.1 ^a	6.9 \pm 0.5 ^a
$^{35}\text{ISW}_{100}$	55 \pm 2.1 ^{b,c}	148 \pm 10 ^{a,b}	35.9 \pm 0.6 ^{a,b}	86.9 \pm 0.6 ^a	6.7 \pm 1.5 ^a
$^{25}\text{ISW}_0$	55 \pm 1.1 ^{b,c}	174 \pm 13 ^a	36.5 \pm 0.3 ^b	84.8 \pm 2.5 ^{a,b}	5.8 \pm 0.9 ^a
$^{25}\text{ISW}_{33}$	52 \pm 1.1 ^c	149 \pm 19 ^{a,b}	34.3 \pm 0.2 ^a	85.9 \pm 0.7 ^{a,b}	6.0 \pm 1.1 ^a
$^{25}\text{ISW}_{66}$	52 \pm 2.0 ^c	122 \pm 4 ^b	35.9 \pm 0.6 ^{a,b}	79.9 \pm 1.3 ^b	6.1 \pm 0.9 ^a
$^{25}\text{ISW}_{100}$	52 \pm 1.1 ^{b,c}	157 \pm 5 ^{a,b}	34.3 \pm 0.5 ^a	84.9 \pm 1.3 ^{a,b}	6.8 \pm 0.6 ^a

Letters as superscript represents significant differences down the column (a, b, c, etc.) (Difference between ionic profiles)

The agar yield showed a strong inverse correlation ($R^2=0.98$, $p < 0.05$) with SGR at the fourth week at 35 ppt. Under culture conditions, the soluble carbohydrate content was strongly correlated to agar yield ($R^2=0.85$, $p < 0.05$). No correlation was observed with gel strength and growth rate but gel strength at 35 ppt showed strong positive correlation ($R^2=0.98$, $p < 0.05$) with gel strength at 25 ppt. In addition, agar yield at 25 ppt showed strong positive correlation ($R^2=0.96$, $p < 0.05$) with the gel strength at 25 ppt. A strong inverse correlation ($R^2=0.80$, $p < 0.05$) was also observed between gel strength and gelling temperature at 35 ppt ($R^2=0.80$, $p < 0.05$) and 25 ppt ($R^2=0.99$, $p < 0.05$) in different ionic profiles. In addition, melting point showed a strong inverse

correlation with the ash content at 35 ppt ($R^2=0.93$, $p<0.05$) and 25 ppt ($R^2=0.99$, $p<0.05$). Ash content also showed a positive correlation ($R^2=0.95$, $p<0.05$) with gel strength and gelling temperature at 25 ppt.

6.4. DISCUSSION

6.4.1. Growth

Results indicate it is technically feasible to culture *G. cliftonii* in all the tested ionic profiles of ISW. Although, previous reports have demonstrated the feasibility of *Gracilaria* species indoor cultivation under different salinity conditions in OW (Friedlander, 1991; Gutknecht, 1965; Ugarte & Santelices, 1992), the effect of different ionic profiles of ISW on growth, chemical composition and agar characteristics of *G. cliftonii* has never been reported.

The SGR of *G. cliftonii* observed ($0.88-1.6\ \%\text{day}^{-1}$) during first week of the experiment was lower compared to *G. chilensis* tank cultured (Ugarte & Santelices, 1992), but was higher than in pond culture as reported by Shang (1976) in Taiwan and Hurtado-Ponce *et al.* (1992) in Philippines. The growth rates of *G. cliftonii* have not been reported in literature therefore the difference in growth rates observed are probably due to the different species of *Gracilaria*.

The occurrence of higher growth rates in the first week of cultivation might be associated with the fact that the seaweeds were collected from natural populations growing in oligotrophic waters with lower nitrogen and phosphorus content. The nutritional status as reflected by chemical composition of seaweeds influences the absorption kinetics of nutrients, thus seaweeds with low nitrogen and phosphorus contents rapidly absorb those nutrients and exhibit faster growth during the initial days of cultivation (DeBoer, 1981; Fujita, 1985; Lobban & Harrison, 1994). The SGR in different ionic profiles at 25 ppt and the lower growth rates observed at 35 ppt indicates that low salinities are adequate for growth of *G. cliftonii*. Similarly, Shang (1976) suggested that the optimum salinity for growing *Gracilaria* species is 25 ppt. Navarro-Angulo and Robledo (1999) also observed a decrease on SGR when *G. cornea* was cultured during extended periods at 35 ppt.

In addition, the results suggest that a two-week acclimatisation period was not adequate for *G. cliftonii*. The SGR on weekly basis of *G. cliftonii* showed a decrease in growth rates for the first four weeks and then an increase in all ionic profiles. Thus, indicating that a longer acclimatisation time is required for *G.*

cliftonii. In contrast, a 2-3 day acclimation period was adequate for *G. tikvahiae* with a salinity range of 16-26 ppt (Lapointe *et al.*, 1984) and a 4-day acclimation period was suggested by Yarish *et al.* (1980) for two estuarine red algae.

On a weekly basis higher SGRs were observed during the third and fifth week after nutrients were provided, suggesting nutrient deprivation in other weeks might have led to relatively lower SGR. The addition of nutrient in the second and fourth week might have contributed to the changes observed in the ionic concentration of the external medium (water) resulting in ionic stress to *G. cliftonii* thus decreasing the growth rates. In addition, weekly maintenance of thalli could have partially contributed to thalli fragmentation and consequent decreased growth rates.

Gutknecht (1965) reported active efflux of Na^+ and active influx of K^+ along with ionic regulation in *G. foliifera*. In addition, he reported that K^+ tends to maintain equilibrium between thalli and water. In the present study, Na^+ and K^+ at the same salinity in different ionic profiles showed a strong positive correlation ($R^2 > 0.9$, $p < 0.05$) between thalli and water, indicating the presence of a mechanism involved in ionic regulation by *G. cliftonii*. In addition, in different ionic profiles similar thalli and water $[\text{K}^+]$ indicates that seaweed tends to maintain an equilibrium of K^+ by active influx from water to thalli which is in accordance to the findings of Gutknecht (1965). The presence of calcium in the medium is necessary for K^+ retention by plants in diluted seawater. Calcium could not be shown to participate directly in K^+ uptake and Na^+ excretion, but is involved in the maintenance of membrane structure (Eppley, 1958a). The resistance of marine algae to hypotonic solutions increases with the calcium content of the medium (Eppley, 1958b) and is correlated with the fact that calcium deficiency causes rapid loss of potassium from the cells (Eppley, 1958a; b). The ratio of divalent ions ($\text{Ca}^{2+}/\text{Mg}^{2+}$) showed a strong correlation between thalli and water supporting the hypothesis that divalent ions is actively transported. Thus, overall changes in $\text{Ca}^{2+}/\text{Mg}^{2+}$, $[\text{Na}^+]$ and $[\text{K}^+]$ in ionic profiles may have induced transient, decreased growth rates in *G. cliftonii*.

Guillard (1962) indicated that the vacuole contains a higher concentration of salt than the medium and serves to excrete salt-thereby conserving water like the salt glands of marine organisms. Therefore, the possibility of ion selectivity and transport may be dependent upon a Na/K activated ATPase of the membrane, which has been demonstrated in different prawn species (Prangnell, 2006; Tantulo & Fotedar, 2007) but is still unknown for any seaweed species. In addition, low $[\text{K}^+]$ in

ISW has shown no significant effect on the growth of *G. cliftonii* in different ionic profiles of ISW indicating that the differences in the SGR in different ionic profiles is due to the concentration of other ions and the ratios formed due to those ions. However, extensive trials will be required for better understanding the effect of the ion transport and uptake mechanism on growth parameters of *G. cliftonii* by keeping the $[K^+]$ constant.

6.4.2. Chemical composition

The present results have shown that the proximate composition of *G. cliftonii* cultured and from natural populations is similar to other seaweeds, that is, rich in non-starch polysaccharides and minerals followed by protein content (Morris, 1974; Syrett, 1962). The protein content of *G. cliftonii* in culture conditions was higher than in the natural population indicating nitrogen assimilation by seaweeds under changed ionic concentrations and ionic stress does occur (Aderhold *et al.*, 1996; Caliceti *et al.*, 2002; Davis *et al.*, 2000; Muse *et al.*, 1999) but may also be related to greater nitrogen and phosphorus availability under experimental conditions. Similarly, the strong inverse correlation of ash content to carbohydrate and protein at different salinities can be attributed to the change in ionic concentrations in the culture environment. Concentrations of most essential elements in *G. cliftonii* are similar to those reported for congeneric species (Freile-Pelegrin & Robledo, 1997b; McDermid & Stuercke, 2003). The constant K^+ and Mg^{2+} concentration in *G. cliftonii* in different water types can be attributed to the efflux and influx of these ions between internal and external media. Various authors have reported heavy metal absorption (up to four times) by different seaweeds indicating that they are naturally bio-absorbent (Friedlander & Dawes, 1984a; Glenn *et al.*, 1996; Ye *et al.*, 2005). The present study confirms that *G. cliftonii* has potential for bioabsorbing purposes as there was approximately four times accumulation of heavy metals. As *Gracilaria* species are important as a food source it is important to consider that the accumulation of these heavy metals could increase the toxicity above food standard levels which is not desirable in food products. Additionally, substitution of heavy metals such as cadmium and manganese in the seaweed media could further challenge its use, thus is necessary to define the final use for which the species is cultivated.

6.4.3. Agar characteristics

In the present study native agar yield of *G. cliftonii* from natural populations was 62.4 %db, which is higher than the reported value of 52 %db (Byrne *et al.*, 2002). The ionic profiles of ISW had a detrimental effect on agar yield and gel strength as compared to natural populations. The decrease in the agar yields under culture conditions could be due to the decrease in soluble carbohydrate content or additional thickening of cell wall carbohydrates in native populations induced by translocational stresses. The agar yield decreased with increasing specific growth rate at 35 ppt and was related to changes in ash and/or insoluble carbohydrate or fibre content of the seaweed, which depends upon the ionic concentration. The dependence of agar yield on proximate composition and its relation to growing conditions have been reported by Ekman, *et al.* (1991). The higher agar yield observed at 35 ppt compared to 25 ppt in ISW₃₃ and ISW₆₆ was caused by changes in agar solubility and/or agar biosynthesis, which may have been altered due to the ion regulation under different ionic profiles of ISW.

The inverse relationship between agar yield and gel strength is well known in *Gracilaria* species (Lahaye & Rochas, 1991) and it is usually attributed to physiological mechanisms (Villanueva & Montaña, 1999). The gel strength of *G. cliftonii* as well as the effect of changing ionic profiles on gel strength has not been reported. However, in the present study, an inverse relation was observed between gel strength and agar yield for *G. cliftonii* from both natural populations and from culture. However, the positive correlation between gel strength and agar yield in different ionic profiles may be due to the altered ionic concentration producing different agar structure.

The gelling temperature of *G. cliftonii* meets the demand of the international market, being within the range 33-38 °C established by *US Pharmacopoeia* compendium of quality tests (Freile-Pelegrin & Murano, 2005). The effects of different ionic profiles on the melting point have not been previously reported. However, the inverse correlation of melting point with ash content explains the effect of different ionic profiles. The inverse relationship between melting point and gelling temperature is well known in seaweeds (Villanueva & Montaña, 1999) and it is usually attributed to physiological mechanisms (Lahaye & Rochas, 1991).

In summary, the ability of *G. cliftonii* to maintain maximum growth rates over a broad range of salinities and different ionic profiles is an important ecological adaptation of this species. The absence of literature on *G. cliftonii* does not provide baseline data for comparison of the growth rates and agar characteristics from culture as well from natural populations. However, the present study shows *G. cliftonii* growth rates are adequate for seaweed aquaculture and can be grown in ocean and inland saline water. *G. cliftonii* showed different growth parameters and agar characteristics in different ionic profiles of ISW. This indicates that growth is not affected by the low $[K^+]$ of ISW but could be due to difference in overall ionic concentration and ionic ratios in these ionic profiles. Therefore, to understand the effect of low $[K^+]$ of ISW on growth and agar characteristics, these ionic profiles should be fortified with potassium.

**CHAPTER 7: EFFECT OF POTASSIUM FORTIFICATION OF
INLAND SALINE WATER ON GROWTH AND AGAR
CHARACTERISTICS**

7.1. INTRODUCTION

In the previous chapter it was identified that the low $[K^+]$ of ISW had no significant effect on the growth of *G. cliftonii*. It was identified that the differences in the concentrations of other ions and the ratios formed in different ionic profiles of ISW might have affected the growth and agar characteristics of *G. cliftonii*. In addition, the differences in the mineral composition of *G. cliftonii* in culture indicated that the growth might have resulted due to ion transport. The negative effect of the low $[K^+]$ of ISW on the survival and growth of prawns is well established (Prangnell, 2006; Tantulo, 2007). However, there is no published information on the effect of $[K^+]$ in OW and ISW for any seaweed. Previous studies on *Gracilaria* and *Porphyra* species have indicated the transport of K^+ from water to plant tissue along with Na^+ and Cl^- without explaining the role of these ions in growth.

The present chapter investigates the effect of K^+ fortification on the chemical composition, growth rates, physicochemical properties and agar characteristics of *G. cliftonii* in ISW.

7.2. MATERIAL AND METHODS

7.2.1. Sample collection

G. cliftonii fronds from the fourth collection were used (Chapter 2) and acclimatised for six weeks in controlled conditions (Chapter 2).

7.2.2. Ionic profiles

Eight ionic profiles were prepared by mixing OW and ISW in different proportions as described in Chapter 6. Ionic profiles, $^{35}ISW_{33}$, $^{35}ISW_{66}$ and $^{35}ISW_{100}$ were K^+ fortified using potassium chloride (analytical grade) solution to obtain an equal $[K^+]$ as in $^{35}ISW_0$ (392 mg.L⁻¹) while $^{25}ISW_{33}$, $^{25}ISW_{66}$ and $^{25}ISW_{100}$ were fortified to obtain an equal $[K^+]$ as in $^{25}ISW_0$ (280 mg.L⁻¹). The concentrations of major ions and ratios for each ionic profile are shown in Table 7.1.

Table 7.1: Ionic composition of potassium fortified inland saline water profiles (mgL^{-1}) and major ionic ratios at salinities 25 ppt and 35 ppt

Ionic profiles	Na⁺	K⁺	Ca²⁺	Mg²⁺	Fe^{2+/3+}	S⁴⁺	Na-K ratio	Ca-Mg ratio
³⁵ ISW ₀	10752	392	392	1232	0.025	2702	27	0.32
³⁵ ISW ₃₃	10644	392	470	1470	0.029	2541	27	0.32
³⁵ ISW ₆₆	10643	392	552	1720	0.033	2406	27	0.32
³⁵ ISW ₁₀₀	10750	392	640	1990	0.037	2295	27	0.32
²⁵ ISW ₀	7680	280	280	880	0.018	1930	27	0.32
²⁵ ISW ₃₃	7603	280	336	1050	0.021	1815	27	0.32
²⁵ ISW ₆₆	7602	280	394	1229	0.024	1719	27	0.32
²⁵ ISW ₁₀₀	7679	280	457	1421	0.027	1639	27	0.32

7.2.3. Experimental design

The experimental design was similar to Chapter 6 except that the trial was conducted for 3 months. An inoculum of 40 g fresh weight of *G. cliftonii* vegetative fronds was used in each container. The biomass was weighed every month to determine SGR and net yield as described in Chapter 2. At the end of every month the samples were weighed and transferred to into containers with fresh nutrients and water with the initial inoculum of 40 g. The excess of seaweed material was weighed, washed and oven dried for later analysis. Nutrients were provided in the form of 20 mL/L of Provasoli Seawater medium (PES) every week. At the end of the experiment, samples from all the containers were washed with fresh water, oven dried for agar extraction and physicochemical properties and freeze dried for chemical composition.

7.2.4. Chemical composition

The chemical composition of dried *G. cliftonii* fronds from indoor culture and natural populations were determined by the method described in Chapter 2.

7.2.5. Physicochemical properties

The physicochemical properties of dried *G. cliftonii* fronds from culture in different ionic profiles were determined by the method described in Chapter 2.

7.2.6. Agar extraction and properties

The agar yield and properties of dried *G. cliftonii* fronds from culture in different ionic profiles were determined by the method described in Chapter 2.

7.3. RESULTS

7.3.1. Growth

Potassium fortification of ISW for the eight ionic profiles used significantly influenced the SGR and net yield of *G. cliftonii* (Table 7.2 and 7.3). Over the entire experiment SGR in different ionic profiles was not influenced by time. The SGR and net yield in ionic profile $^{25}\text{ISW}_{33}$ were significantly lower ($p < 0.05$) than $^{25}\text{ISW}_{100}$ for the first month. During the second month, different ionic profiles had no influence ($p > 0.05$) on SGR and net yield of *G. cliftonii*. After the third month, SGR in $^{25}\text{ISW}_{33}$ was significantly lower ($p < 0.05$) than in $^{35}\text{ISW}_0$, $^{35}\text{ISW}_{33}$ and $^{35}\text{ISW}_{100}$. However, net yield in $^{25}\text{ISW}_{33}$ was significantly lower ($p < 0.05$) than in $^{35}\text{ISW}_{33}$ and $^{35}\text{ISW}_{100}$.

Table 7.2: Specific growth rate (Mean \pm S.E.) (%day⁻¹) of *Gracilaria cliftonii* in different ionic profiles during three months of culture

Ionic profiles	Month		
	1	2	3
$^{35}\text{ISW}_0$	$1.11 \pm 0.1^{a,b}$	1.10 ± 0.2^a	0.93 ± 0.1^a
$^{35}\text{ISW}_{33}$	$0.92 \pm 0.1^{a,b}$	0.96 ± 0.2^a	0.96 ± 0.1^a
$^{35}\text{ISW}_{66}$	$1.02 \pm 0.1^{a,b}$	0.85 ± 0.2^a	$0.81 \pm 0.1^{a,b}$
$^{35}\text{ISW}_{100}$	$1.00 \pm 0.2^{a,b}$	1.03 ± 0.1^a	0.95 ± 0.2^a
$^{25}\text{ISW}_0$	$0.93 \pm 0.1^{a,b}$	0.76 ± 0.1^a	$0.61 \pm 0.1^{a,b}$
$^{25}\text{ISW}_{33}$	0.79 ± 0.1^a	0.75 ± 0.2^a	0.52 ± 0.2^b
$^{25}\text{ISW}_{66}$	$1.00 \pm 0.2^{a,b}$	0.82 ± 0.2^a	$0.57 \pm 0.1^{a,b}$
$^{25}\text{ISW}_{100}$	1.24 ± 0.1^b	0.88 ± 0.3^a	$0.73 \pm 0.1^{a,b}$

Letters as superscript represent significant differences down the column (a, b, c) (Difference between ionic profiles)

Table 7.3: Net yield ($\text{g.m}^{-2}\text{day}^{-1}$) (Mean \pm S.E.) of *Gracilaria cliftonii* in different ionic profile during three months of culture

Ionic profiles	Month		
	1	2	3
³⁵ ISW ₀	$3.54 \pm 0.4^{a,b}$	3.45 ± 0.6^a	$2.87 \pm 0.4^{a,b}$
³⁵ ISW ₃₃	$2.82 \pm 0.3^{a,b}$	3.00 ± 0.6^a	2.97 ± 0.4^a
³⁵ ISW ₆₆	$3.22 \pm 0.5^{a,b}$	2.61 ± 0.6^a	$2.44 \pm 0.2^{a,b}$
³⁵ ISW ₁₀₀	$3.16 \pm 0.6^{a,b}$	3.23 ± 0.3^a	3.00 ± 0.9^a
²⁵ ISW ₀	$2.89 \pm 0.3^{a,b}$	2.28 ± 0.3^a	$1.78 \pm 0.2^{a,b}$
²⁵ ISW ₃₃	2.39 ± 0.5^a	2.33 ± 0.8^a	1.55 ± 0.5^b
²⁵ ISW ₆₆	$3.13 \pm 0.6^{a,b}$	2.55 ± 0.8^a	$1.67 \pm 0.1^{a,b}$
²⁵ ISW ₁₀₀	4.03 ± 0.5^b	2.83 ± 1.1^a	$2.18 \pm 0.4^{a,b}$

Letters as superscript represent significant differences down the column (a, b, c) (Difference between ionic profiles)

7.3.2. Chemical composition

The chemical composition of *G. cliftonii* from both natural populations and culture consists mainly of carbohydrates followed by ash and protein (Table 7.4). The carbohydrate content of *G. cliftonii* in the ionic profile ³⁵ISW₃₃ was significantly higher ($p < 0.05$) than in ³⁵ISW₆₆. The ash content of *G. cliftonii* natural population and ³⁵ISW₆₆ was significantly higher ($p < 0.05$) than other ionic profiles. The protein content of *G. cliftonii* significantly increased ($p < 0.01$) under culture conditions as compared to the natural population. The protein content of *G. cliftonii* in ²⁵ISW₃₃ and ²⁵ISW₁₀₀ was significantly higher ($p < 0.05$) than in ³⁵ISW₀, ³⁵ISW₃₃ and ³⁵ISW₁₀₀.

Table 7.4: Proximate composition (%db) (Mean \pm S.E.) of *Gracilaria cliftonii* from natural population and in different ionic profiles after three months of culture

Ionic profiles	Ash	Protein	Carbohydrate
Natural population	33.6 \pm 1.4 ^a	12.9 \pm 1.0 ^a	52.6 \pm 2.0 ^{a,b}
³⁵ ISW ₀	29.4 \pm 2.4 ^{a,b}	17.3 \pm 0.9 ^b	53.38 \pm 2.2 ^{a,b}
³⁵ ISW ₃₃	24.4 \pm 1.9 ^b	17.5 \pm 0.8 ^b	58.10 \pm 2.4 ^a
³⁵ ISW ₆₆	33.9 \pm 2.9 ^a	18.2 \pm 1.2 ^{b,c}	47.85 \pm 4.0 ^b
³⁵ ISW ₁₀₀	25.3 \pm 3.1 ^b	17.3 \pm 0.4 ^b	57.35 \pm 2.8 ^{a,b}
²⁵ ISW ₀	24.7 \pm 3.1 ^b	20.4 \pm 1.4 ^{b,c}	54.93 \pm 2.4 ^{a,b}
²⁵ ISW ₃₃	24.6 \pm 2.9 ^b	21.3 \pm 1.5 ^c	54.10 \pm 3.9 ^{a,b}
²⁵ ISW ₆₆	26.3 \pm 3.1 ^b	20.2 \pm 1.2 ^{b,c}	53.55 \pm 3.9 ^{a,b}
²⁵ ISW ₁₀₀	24.0 \pm 4.6 ^b	20.5 \pm 0.9 ^c	55.57 \pm 5.1 ^{a,b}

Letters as superscript represent significant differences down the column (a, b, c) (Difference between ionic profiles)

The variation in mineral composition of *G. cliftonii* from both natural populations and culture is shown in Table 7.5. In natural populations and culture, the order of concentration of different elements was K>Na>S>Mg>Ca>P>Fe. The [P] of *G. cliftonii* in culture profiles except ²⁵ISW₁₀₀ was significantly higher (p<0.05) than the natural population. In addition, [P] of *G. cliftonii* in ²⁵ISW₃₃ was significantly higher (p<0.05) than in ³⁵ISW₆₆, ³⁵ISW₁₀₀, ²⁵ISW₀ and ²⁵ISW₁₀₀. The [K⁺] of *G. cliftonii* in culture profiles decreased significantly (p<0.05) as compared to natural population. However, different ionic profiles had no influence (p>0.05) on [K⁺] of *G. cliftonii*. [Mg²⁺] of *G. cliftonii* in ³⁵ISW₆₆ was significantly higher (p<0.05) than in ²⁵ISW₁₀₀, ²⁵ISW₃₃, ²⁵ISW₆₆ and ²⁵ISW₁₀₀. Iron content of *G. cliftonii* from natural population and in ³⁵ISW₁₀₀, ³⁵ISW₃₃ was significantly lower (p<0.05) than in ³⁵ISW₆₆. [Ca²⁺] of *G. cliftonii* from natural population and in ionic profiles at 35 ppt was significantly lower (p<0.05) than in ionic profiles at 25 ppt. Ionic profiles had no influence (p>0.05) on [S] of *G. cliftonii*.

Table 7.5: Mineral composition (%db) (Mean \pm S.E.) of *Gracilaria cliftonii* from natural population and in culture after three months of culture

Ionic profiles	Na	K	Ca	Mg	Fe	S
Natural population	$5.0 \pm 0.2^{a,b}$	9.5 ± 0.4^a	0.6 ± 0.0^a	$1.1 \pm 0.1^{a,b}$	0.015^a	2.0 ± 0.2^a
$^{35}\text{ISW}_0$	5.5 ± 1.5^a	6.6 ± 1.2^b	0.5 ± 0.1^a	$1.3 \pm 0.2^{a,b}$	0.019^a	2.3 ± 0.0^a
$^{35}\text{ISW}_{33}$	$4.4 \pm 0.9^{a,b}$	6.9 ± 0.8^b	0.6 ± 0.1^a	$1.3 \pm 0.2^{a,b}$	0.021^a	2.2 ± 0.1^a
$^{35}\text{ISW}_{66}$	6.4 ± 0.4^a	$7.1 \pm 0.9^{a,b}$	0.6 ± 0.1^a	1.5 ± 0.1^a	0.045^b	2.1 ± 0.1^a
$^{35}\text{ISW}_{100}$	$4.8 \pm 0.7^{a,b}$	6.8 ± 0.9^b	0.6 ± 0.1^a	$1.3 \pm 0.2^{a,b}$	$0.024^{a,b}$	2.0 ± 0.2^a
$^{25}\text{ISW}_0$	3.9 ± 1.1^b	6.0 ± 0.5^b	1.1 ± 0.2^b	1.0 ± 0.1^b	$0.026^{a,b}$	2.2 ± 0.1^a
$^{25}\text{ISW}_{33}$	2.6 ± 0.6^b	$7.1 \pm 0.8^{a,b}$	2.1 ± 0.8^b	1.1 ± 0.1^b	$0.034^{a,b}$	2.3 ± 0.2^a
$^{25}\text{ISW}_{66}$	3.6 ± 0.5^b	$7.6 \pm 0.5^{a,b}$	1.4 ± 0.9^b	1.2 ± 0.1^b	$0.028^{a,b}$	2.2 ± 0.2^a
$^{25}\text{ISW}_{100}$	3.1 ± 0.3^b	6.5 ± 1.0^b	2.7 ± 0.8^b	1.0 ± 0.0^b	$0.031^{a,b}$	1.9 ± 0.1^a

Letters as superscript represent significant differences down the column (a, b, c) (Difference between ionic profiles)

The N-P, Na-K, Ca-Mg and C-N ratios of *G. cliftonii* from natural populations and indoor culture are shown in Table 7.6. The N-P ratio in the ionic profile $^{25}\text{ISW}_0$ and $^{25}\text{ISW}_{100}$ were significantly higher ($p < 0.05$) than in $^{35}\text{ISW}_0$, $^{35}\text{ISW}_{33}$ and $^{35}\text{ISW}_{66}$. The Na-K ratio in $^{35}\text{ISW}_0$ was significantly higher ($p < 0.01$) than from natural population and in $^{25}\text{ISW}_{33}$, $^{25}\text{ISW}_{66}$, $^{25}\text{ISW}_{100}$. The Ca-Mg ratio from natural population and in $^{35}\text{ISW}_0$, $^{35}\text{ISW}_{33}$, $^{35}\text{ISW}_{100}$ were significantly lower ($p < 0.01$) than in $^{25}\text{ISW}_{33}$ and $^{35}\text{ISW}_0$. The C-N ratio from natural population and in $^{35}\text{ISW}_0$, $^{35}\text{ISW}_{33}$ were significantly higher ($p < 0.05$) than in $^{25}\text{ISW}_0$ and $^{25}\text{ISW}_{33}$.

Table 7.6: Important element ratios (Mean \pm S.E.) of *Gracilaria cliftonii* from natural populations and in different ionic profiles

Ionic profiles	N-P ratio	Na-K ratio	Ca-Mg ratio	C-N ratio
Natural population	13.7 \pm 1.0 ^{a,b}	0.5 \pm 0.0 ^b	0.5 \pm 0.0 ^a	25.9 \pm 2.9 ^a
³⁵ ISW ₀	10.6 \pm 1.1 ^a	1.1 \pm 0.5 ^a	0.4 \pm 0.0 ^a	24.8 \pm 1.9 ^a
³⁵ ISW ₃₃	11.4 \pm 1.0 ^a	0.6 \pm 0.1 ^{a,b}	0.5 \pm 0.0 ^a	24.3 \pm 1.4 ^a
³⁵ ISW ₆₆	11.6 \pm 0.6 ^a	1.0 \pm 0.2 ^{a,b}	1.5 \pm 1.1 ^{a,b}	21.3 \pm 2.1 ^{a,b}
³⁵ ISW ₁₀₀	12.8 \pm 1.2 ^{a,b}	0.7 \pm 0.1 ^{a,b}	0.5 \pm 0.1 ^a	24.0 \pm 0.6 ^{a,b}
²⁵ ISW ₀	14.5 \pm 0.9 ^b	0.7 \pm 0.2 ^{a,b}	1.3 \pm 0.4 ^{a,b}	19.8 \pm 1.7 ^b
²⁵ ISW ₃₃	12.6 \pm 0.7 ^{a,b}	0.4 \pm 0.1 ^b	2.0 \pm 0.8 ^b	19.3 \pm 2.0 ^b
²⁵ ISW ₆₆	13.4 \pm 0.3 ^{a,b}	0.5 \pm 0.0 ^b	1.1 \pm 0.6 ^{a,b}	20.0 \pm 1.8 ^{a,b}
²⁵ ISW ₁₀₀	14.8 \pm 0.7 ^b	0.5 \pm 0.1 ^b	2.6 \pm 0.8 ^b	20.0 \pm 1.5 ^{a,b}

Letters as superscript represents significant differences down the column (a, b, c) (Difference between ionic profiles)

7.3.3. Physicochemical properties

Fortification with K⁺ and ionic profile of ISW had no influence on the WRC and ORC of *G. cliftonii* (Table 7.7).

Table 7.7: Water and oil retention capacity (g/g) (Mean \pm S.E.) of *Gracilaria cliftonii* from natural populations and in different ionic profiles after three months of culture

Ionic profiles	Water retention capacity	Oil retention capacity
Natural population	6.6 \pm 1.6 ^a	3.1 \pm 0.5 ^a
³⁵ ISW ₀	6.0 \pm 0.7 ^a	3.3 \pm 0.5 ^a
³⁵ ISW ₃₃	6.7 \pm 0.4 ^a	3.4 \pm 0.4 ^a
³⁵ ISW ₆₆	7.2 \pm 0.3 ^a	3.4 \pm 0.9 ^a
³⁵ ISW ₁₀₀	6.2 \pm 0.4 ^a	4.1 \pm 0.1 ^a
²⁵ ISW ₀	6.9 \pm 0.3 ^a	3.8 \pm 0.6 ^a
²⁵ ISW ₃₃	6.1 \pm 0.2 ^a	2.7 \pm 0.5 ^a
²⁵ ISW ₆₆	6.9 \pm 0.6 ^a	3.4 \pm 1.1 ^a
²⁵ ISW ₁₀₀	6.2 \pm 0.5 ^a	4.1 \pm 1.1 ^a

Letters as superscript represents significant differences down the column (a, b, c) (Difference between ionic profiles)

7.3.4. Agar characteristics

The agar yield and properties of *G. cliftonii* cultured in K⁺ fortified ISW and different ionic profiles are shown in Table 7.8. Agar yield of *G. cliftonii* from natural populations and in ³⁵ISW₃₃ were significantly higher (p<0.05) than in ²⁵ISW₁₀₀. The ionic profiles of ISW used had a detrimental effect on the agar properties viz. gel strength, melting point and gelling temperature of agar compared to the agar extracted from natural populations. The gel strength of the agar from natural populations was significantly higher (p<0.01) than the culture profiles. In addition, gel strength of agar in ³⁵ISW₀ was significantly higher (p<0.05) than in other profiles. The melting point of agar decreased significantly (p<0.05) in culture profiles ³⁵ISW₀, ³⁵ISW₃₃, ³⁵ISW₆₆, ²⁵ISW₃₃, ²⁵ISW₁₀₀ as compared to natural populations. Gelling temperature of agar from natural populations and in ³⁵ISW₁₀₀ were significantly higher (p<0.05) than in ³⁵ISW₀, ³⁵ISW₃₃ and ³⁵ISW₆₆. The sulphate content of agar from *G. cliftonii* in ³⁵ISW₀ and ²⁵ISW₁₀₀ were significantly higher (p<0.05) than in other profiles but showed no significant difference from natural populations.

Table 7.8: Agar characteristics (Mean \pm S.E.) of *Gracilaria cliftonii* from natural populations (NP) and in different ionic profiles after three months of culture

Ionic profiles	Agar yield (%db)	Gel strength (g. cm⁻²)	Melting point (°C)	Gelling temp. (°C)	Sulphate content (%db)
NP	58.2 \pm 0.6 ^a	161.8 \pm 3.6 ^a	86.8 \pm 0.5 ^a	35.2 \pm 0.6 ^a	5.6 \pm 0.1 ^{a,b}
³⁵ISW₀	57.1 \pm 2.0 ^{a,b}	135.6 \pm 0.6 ^b	81.0 \pm 1.3 ^b	32.7 \pm 0.7 ^b	6.1 \pm 0.3 ^a
³⁵ISW₃₃	58.6 \pm 0.7 ^a	122.9 \pm 1.3 ^c	82.2 \pm 2.1 ^b	32.9 \pm 0.3 ^b	4.5 \pm 0.1 ^b
³⁵ISW₆₆	53.8 \pm 2.8 ^{a,b}	121.5 \pm 7.7 ^c	82.4 \pm 1.9 ^b	32.1 \pm 1.3 ^b	4.8 \pm 0.5 ^b
³⁵ISW₁₀₀	52.1 \pm 5.2 ^{a,b}	120.4 \pm 3.9 ^c	83.2 \pm 0.3 ^{a,b}	35.1 \pm 0.4 ^a	4.8 \pm 0.5 ^b
²⁵ISW₀	55.4 \pm 1.1 ^{a,b}	118.0 \pm 1.9 ^c	83.4 \pm 0.8 ^{a,b}	33.8 \pm 0.7 ^{a,b}	5.0 \pm 0.2 ^b
²⁵ISW₃₃	57.8 \pm 2.4 ^{a,b}	115.8 \pm 1.6 ^c	80.7 \pm 1.6 ^b	33.4 \pm 0.5 ^{a,b}	5.2 \pm 0.6 ^b
²⁵ISW₆₆	57.3 \pm 1.2 ^{a,b}	120.1 \pm 4.6 ^c	83.1 \pm 0.4 ^{a,b}	34.1 \pm 0.8 ^{a,b}	4.6 \pm 0.2 ^b
²⁵ISW₁₀₀	50.9 \pm 2.3 ^b	119.4 \pm 1.2 ^c	82.4 \pm 1.0 ^b	34.2 \pm 1.0 ^{a,b}	5.9 \pm 0.3 ^a

Letters as superscript represents significant differences down the column (a, b, c, etc.) (Difference between ionic profiles)

7.3.5. Correlations

G. cliftonii cultured in potassium fortified ISW with different ionic profiles showed significant correlations (R^2) ($p < 0.05$) between growth parameters, chemical composition and agar properties. SGR and net yield showed strong positive correlation with C-N ratio ($R^2 = +0.88$ and $R^2 = +0.89$, respectively) and ash content ($R^2 = +1.00$ and $R^2 = +0.98$). However, ash content was negatively correlated ($R^2 = -0.77$) with the carbohydrate content. Protein content was negatively correlated with the Na^+ ($R^2 = -0.63$) and Mg^{2+} ($R^2 = -0.63$) content of *G. cliftonii*. The N-P ratio was negatively correlated ($R^2 = -0.63$) with Mg^{2+} content while Ca-Mg ratio showed negative correlation ($R^2 = -0.67$) with C-N ratio. Strong positive correlations were observed between Na^+ and Mg^{2+} ($R^2 = +0.73$), P and S ($R^2 = +0.70$) and Ca^{2+} and $\text{Fe}^{2+/3+}$ ($R^2 = +0.70$) contents of *G. cliftonii*. Agar yield showed strong positive correlation with P content ($R^2 = +0.78$) and S content ($R^2 = +0.85$).

7.4. DISCUSSION

In the present study potassium fortification of ISW for indoor culture of *G. cliftonii* affected growth parameters, chemical composition, physicochemical properties and agar characteristics. At the end of 3 months of the experiment, K^+ fortification significantly increased the SGR and net yield of *G. cliftonii* cultured with the ionic profiles ISW₁₀₀ and ISW₃₃ at 35 ppt as compared to ISW₃₃ at 25 ppt. In addition, the higher SGR and net yield in K fortified ionic profiles achieved at 35 ppt as compared to 25 ppt indicates that lower salinity ISW has a negative effect on the growth of *G. cliftonii*. Shang (1976) suggested culturing *Gracilaria* species at 25 ppt salinity while Navarro-Angulo & Robledo (1999) suggested culturing *G. cornea* at salinity less than 35 ppt. The difference in the results could be due to the difference in ionic profiles between OW and ISW.

The observed SGR (0.52-0.95 %day⁻¹) in K^+ fortified ISW ionic profiles was higher as compared to the ionic profiles without fortification (0.16-0.38 %day⁻¹, Chapter 7) indicating its role in the growth of *G. cliftonii*. The higher SGR and net yield in K^+ fortified ionic profiles is due to the addition of potassium as shown in Chapter 7. In addition, K^+ is required for nitrogen absorption and storage in seaweed and might have enhanced nutrient absorption, thus increasing the SGR and net yield (Lobban & Harrison, 1994).

The variation in SGR and net yield of *G. cliftonii* cultured in different ionic profiles could be due to the change in concentration of monovalent ions viz. Na^+ , K^+ and Cl^- , divalent ions Ca^{2+} , Mg^{2+} and Fe^{2+} or the trivalent ion Fe^{3+} . Monovalent, divalent and trivalent ions have not been reported to be limiting nutrients for seaweed growth in the marine environment as compared to N and P but their uptake rate is associated with osmoregulatory processes in seaweeds (Reed, 1990). The role of K^+ in ionic relations in seaweeds is non-specific as it is only one of the several monovalent cations involved in osmoregulation. K^+ has a more specific role as an enzyme activator and in many protein syntheses. In addition, enzymes do not act efficiently in the absence of K^+ . It is known to bind ionically to pyruvate kinase which is essential in respiration and carbohydrate metabolism (Lobban & Harrison, 1994). Various studies conducted on the transport of monovalent ions in different seaweed species (Chapter 1) indicated the active influx and efflux of these ions (Gutknecht, 1965; 1966; MacRobbie, 1962; Reed & Collins, 1980; Ritchie & Larkum, 1984a; b). $[K^+]$ of the thalli was similar for all the tested ionic profiles, (6-

7.6 %db) but was lower compared to natural populations. Only $[\text{Na}^+]$ of thalli was similar in culture and natural population. The similar $[\text{K}^+]$ and $[\text{Na}^+]$ of thalli and water for all the ionic profiles indicates that *G. cliftonii* tends to maintain the equilibrium of K^+ and Na^+ by active influx of K^+ and Na^+ from water to thalli. Gutknecht (1965) reported active efflux of Na^+ and active influx of K^+ due to ionic regulation to maintain equilibrium between thalli and water in *G. foliifera*.

Calcium is important to all organisms for maintenance of cellular membranes. Along with other divalent cations it is responsible for ATPase activity and is an important cofactor in ion transport in seaweeds (Lobban & Harrison, 1994). The lower SGR and net yield in different ionic profiles at 25 ppt could be due to the increase in $[\text{Ca}^{2+}]$ as compared to 35 ppt. The presence of calcium in the medium is necessary for K^+ retention by plants in diluted seawater (Lobban & Harrison, 1994). Calcium could not be shown to participate directly in K^+ uptake and Na^+ excretion, but is involved in the maintenance of membrane structure (Eppley, 1958a). The resistance of seaweeds to hypotonic solutions increases with the calcium content of the medium and is correlated with the fact that calcium deficiency causes rapid loss of potassium from the cells (Eppley, 1958a; b). Magnesium is the second important divalent ion and is an essential component of chlorophyll. It is a cofactor and activator in many reactions such as ion transport, nitrate reduction, sulphate reduction and phosphate transfer (Lobban & Harrison, 1994). Similar $[\text{Mg}^{2+}]$ in thalli from natural populations and culture indicates that it was not directly affecting the SGR and net yield but may have been indirectly assisting ion transport and phosphate transfer. Also, Mg is reported to link enzymes and substrates together in reactions involving PO_4 transfer from ATP (Lobban & Harrison, 1994).

The primary transport reaction in which ATP is consumed is the transport of H^+ across cell membranes by H^+ pumping ATPases. The resulting H^+ electrochemical potential gradients are consumed in driving secondary ions via coupled transport. Couple transport may arise from the transport of different ions at different sites on a given carrier in opposite directions or the same direction (Leon, 1963). In many animal cells transport generally takes place by cotransport of Na^+ rather than H^+ . Similarly, in plant cells it is assumed that there is transport of Na^+ as seawater is high in Na^+ and low in H^+ . Guillard (1962) indicated that the vacuole of marine algae contains a higher concentration of salt than the external medium and serves to excrete salt, thereby conserving water like the salt glands of marine organisms.

Therefore, the possibility of ion selectivity and transport may be dependent upon a Na/K activated ATPase of the membrane, which has been demonstrated in different prawn species (Prangnell, 2006; Tantulo & Fotedar, 2007). The isolation of membrane ATPases and the correlation between ion fluxes and ATPases activity suggests that ATP is the primary source for ion transport in seaweeds (Lobban & Harrison, 1994).

Chemical factors such as nutrient uptake are affected by the ionic and molecular form of the element which might have affected the SGR and net yield (DeBoer, 1981). In the present study N was provided in the form of nitrate but the alteration of ionic concentration (higher at 35 ppt) might have affected the uptake rate of nutrients at low salinity (DeBoer, 1981). In seaweeds, N in the form of ammonium is often taken up more rapidly than nitrate, urea or amino acids and is influenced by the concentration of ions in the medium (Hanisak, 1990; Lapointe *et al.*, 1984; Navarro-Angulo & Robledo, 1999). In addition, high concentrations of Ca^{2+} , Na^{+} , Mg^{2+} and K^{+} are reported to inhibit PO_4^{2-} uptake in many seaweeds (Lobban & Harrison, 1994).

Other factors which could have affected the SGR and net yield of *G. cliftonii* in potassium fortified ionic profiles are surface area to volume ratio, hair formation, type of tissue, age of plant, nutritional history and interplant variability (Craigie & Wen, 1984; Lobban & Harrison, 1994) but these were not analysed in the present study. In addition, cutting the thallus to produce tissue segments (inoculum) could also have influenced the uptake rate of nutrients and flow of ions, either through wounding response and increased respiration or by elimination of the translocation system (Hatcher, 1977).

The proximate composition of *G. cliftonii* in present study is similar to other seaweeds *viz.* rich in non-starch polysaccharides and minerals followed by protein (Galland-Irmouli *et al.*, 1999; McDermid & Stuercke, 2003; Norziah & Ching, 2000; Wong & Cheung, 2000). Concentrations of most essential elements in *G. cliftonii* are similar to those reported for congeneric species (Freile-Pelegrin & Robledo, 1997b; McDermid & Stuercke, 2003). The decrease in ash content in culture conditions as compared to natural populations could be due to the absorption of minerals as seaweeds are reported to be good bioabsorbers. The protein content of *G. cliftonii* in culture conditions was higher than in the natural populations indicating nitrogen assimilation due to changes in ion concentrations and to ionic stress (Aderhold *et al.*,

1996; Caliceti *et al.*, 2002; Davis *et al.*, 2000; Muse *et al.*, 1999). In addition, high protein content in ISW₃₃ at 25 ppt as compared to 35 ppt could be due to high nitrogen absorption under salinity stress.

Agar yield of *G. cliftonii* in the present study from natural populations and culture was higher than the reported value of 52 %db (Byrne *et al.*, 2002). The agar yield is also higher than from other *Gracilaria* species (16 to 47 %) (Doty *et al.*, 1983; Freile-Pelegrin & Murano, 2005; Marinho-Soriano, 2001; Marinho-Soriano & Bourret, 2005; Santos & Doty, 1983). This could be due to the difference in species, environmental factors, culture conditions and agar extraction methods (Armisen, 1995; Armisen & Galatas, 1987; Byrne *et al.*, 2002). The decrease in the agar yields in the ionic profile ²⁵ISW₁₀₀ as compared to the natural population and ³⁵ISW₃₃ could be due to the influence of ion transport. Polysaccharides have sulphate, carboxyl and phosphate groups from which protons can dissociate leaving a net negative charge of these compounds in the cell wall. These macromolecules act as cation exchangers and consequently large number of cations can be absorbed from the environment (Lobban & Harrison, 1994). Mg being a divalent cation could affect the agar yield as it is important in binding charged polysaccharide chains. Differences in agar yield could also be due to changes in agar solubility and/or agar biosynthesis along with structural changes due to ion regulation under different ionic profiles of ISW (Craigie & Jurgens, 1989; Duckworth & Yaphe, 1971; Lahaye & Yaphe, 1988). The detrimental effect on the agar properties viz. decrease in gel strength, melting point and gelling temperature and increase in sulphate content in culture as compared to natural populations could be due to the influence of ions on agar structure. Increase in phosphorus content in culture might have affected the structure of agar in culture conditions as the properties showed strong correlation with mineral contents.

SGR and net yield are reported to be influenced by the C-N ratio as it affects nitrogen absorption (Andr  a *et al.*, 2001; Lapointe, 1985; Lignell & Peders  n, 1989). When *Gracilaria foliifera* was grown under N limited conditions the C:N ratio in the thallus was <10 and the plant showed higher rates of ammonium uptake at given ammonium concentrations than did plants that were not N limited (C:N ratio <10) (Lapointe & Duke, 1984). The strong correlation between SGR and C-N ratio at 35 ppt could have facilitated the growth parameters of *G. cliftonii* resulting in higher SGR and net yield in ionic profiles at 35 ppt than at 25 ppt. In addition, the positive correlation of ash content to SGR and net yield in different ionic profiles indicates

the absorption of ions by *G. cliftonii*, resulting in increase in biomass which is a measure of SGR and net yield. Ions like Na^+ , Ca^{2+} and Mg^{2+} also facilitate nutrient absorption and might have increased the SGR and net yield of *G. cliftonii*. SGR and net yield variation could be due to difference in nutrient absorption due to the presence of ions as plant N content was negatively correlated with Na^+ and Mg^{2+} . The negative correlation between the ash and carbohydrate content of *G. cliftonii* in different ionic profiles could be due to the binding of elements in the carbohydrate molecules. This is supported by the increase in agar yield with increase in phosphorus and sulphate content of the seaweed (Duckworth & Yaphe, 1971; Young *et al.*, 1971). The inverse relationship between agar yield and sulphate content is well known in *Gracilaria* species (Lahaye & Rochas, 1991) and it is usually attributed to physiological mechanisms (Villanueva & Montaña, 1999). This relation was also observed in the present study and could be due to the altered ionic concentrations.

In summary, K^+ fortification of ionic profiles of ISW significantly increased the growth rates of *G. cliftonii*. The SGR and net yield obtained over a period of three months indicate it is a slow growing species. In the present study, K^+ fortification at lower salinity had an adverse effect on SGR and net yield which contradicts previous findings for other species (Navarro-Angulo & Robledo, 1999; Shang, 1976). The absence of literature on *G. cliftonii* does not provide satisfactory comparison of the growth rates and agar characteristics in culture as these attributes are species specific. However, the present study shows *G. cliftonii* growth rates are adequate for ISW aquaculture when the ISW is fortified with K^+ . However, ion transport and its effect on growth parameters and agar characteristics requires further investigation in order to provide better understanding of the mechanisms involved in *G. cliftonii* ion regulation.

**CHAPTER 8: INFLUENCE OF ALKALI TREATMENT OF
GRACILARIA CLIFTONII LIFE STAGES ON AGAR YIELD AND
QUALITY**

8.1. INTRODUCTION

In the Chapters 4 to 7, it was observed that the culture conditions influence the agar characteristics of *G. cliftonii*. Literature reports different agar extraction methods for different *Gracilaria* species (Chapter 1) and numerous studies have shown that agar production and quality are influenced by extrinsic factors like season and environmental factors (Bird, 1988; Christiaen *et al.*, 1987; Hoyle, 1978; Luhan, 1992). *Gracilaria* species harvested from natural populations provide most of the world's agar production (Santelices & Doty, 1989). However during the last decade a depletion of the natural stocks has been observed due to their over exploitation (Marinho-Soriano *et al.*, 1999).

Agar content and gel strength of *Gracilaria* species is also influenced by intrinsic factors like life stage (Whyte *et al.*, 1981) (Chapter 1). Penniman (1977) observed differences in agar yield between the reproductive (carposporophyte, tetrasporophyte) and vegetative stages of *G. foliifera*. However, Hoyle (1978) reported no significant differences in yield or gel strength between the stages of *G. bursapastoris* and *G. coronopifolia*.

The agar properties depend on its structure, particularly the number and location of sulphate groups in the polysaccharide chain (Andriamanananatony *et al.*, 2007; Craigie & Jurgens, 1989; Duckworth & Yaphe, 1971; Lahaye & Rochas, 1991). Melting point is affected by the pyruvic acid content (Young *et al.*, 1971) and gelling temperature is related to the methoxyl content and its location in the agar structure (Guisseley, 1970; Rebello *et al.*, 1997). Agar can be characterized by its content of sulphate and other constituents as reported by Asare (1980a) and Zanolungo (1980). The types and amounts of chemical substituents (methoxyl and sulphate) depend not only on the species (Bird & Hinson, 1992; Cote & Hanisak, 1986; Hurtado-Ponce & Umezaki, 1988; Levy *et al.*, 1990) but also on seasonal and physiological factors (Craigie & Wen, 1984; Lahaye & Yaphe, 1988; Patwary & van der Meer, 1983) and life stages of the seaweed (Whyte *et al.*, 1981).

Gracilaria species produce agars with low yield and gel strength due to high sulphate concentrations (Craigie & Jurgens, 1989). However, the gel properties of

many *Gracilaria* agars can be improved by alkali treatment before agar extraction. This process converts L-galactose-6-sulphate to 3,6-anhydro-L-galactose (Duckworth & Yaphe, 1971), which is responsible for the enhancement of the gel forming ability. This pre-treatment must be adapted for each *Gracilaria* species modifying variables like temperature and alkali concentration to obtain maximum desulphation and avoid yield losses that this process can cause (Armisen & Galatas, 1987). The response to the alkali pre-treatment process differs between *Gracilaria* species and specific variables like temperature and alkali concentration can improve the agar quality (Freile-Pelegri & Murano, 2005).

Harvesting of seaweed from natural populations for production of agar during the reproductive periods may affect them considerably. Thus knowledge of quality of agar for different life stages is important when choosing the best period to harvest natural stocks (Marinho-Soriano *et al.*, 1999). This chapter aims to determine the agar characteristics and the effect of alkali treatment and heating time of agar of *G. cliftonii* life stages.

8.2. MATERIAL AND METHODS

8.2.1. Sample and alkali preparation

G. cliftonii were identified and grouped into three life stages (tetrasporophyte, carposporophyte and vegetative) as described in Chapter 3. The fronds were washed with tap water to remove sand and mud and epiphytes were manually removed. Each life stage was oven dried for 8 h at 60 °C and stored in sealed plastic bags until agar extraction. Two concentrations of alkali 0.3 and 0.5 % were prepared by dissolving 3 g and 5 g of analytical grade sodium hydroxide (Sigma®) respectively in 1 L of distilled water.

8.2.2. Alkali treatment of different stages

G. cliftonii samples with 5 g dry weight were used for each life stage *viz.* carposporophyte, tetrasporophyte and vegetative. These samples were soaked for 3 h in 200 mL of 0.3 and 0.5 % alkali solution respectively at room temperature to hydrate the seaweeds in triplicates. The samples without alkali treatment were soaked in 200 mL of distilled water and were used as a control. All the samples were divided into two groups. In the first group sample soaking was followed by removal of excess alkali and agar extraction. In the second group, sample soaking was followed by 1 h heating in a water bath at 70 °C, alkali removal and agar extraction.

8.2.3. Agar extraction

After alkali treatment, the sample was washed with running tap water for 1 h to remove excess alkali. In both groups (with or without heating), extraction was carried out by boiling the sample for 2.5 h in 250 mL of distilled water at 7.0-7.5 pH. The extracts were filtered using three-ply cheesecloth and transferred to plastic containers (500 mL). The filtrate was allowed to gel at room temperature, frozen overnight and then thawed. Finally, the agar was oven dried for 24 h at 60 °C, cooled and weighed to calculate percent agar yield on a dry weight basis (%db). The overall process is shown in Appendix 3.

8.2.4. Agar loss

To quantify agar loss, the samples from the second group (1 h heating) were used. The soaking solution (water and alkali) was transferred to plastic containers, allowed to gel at room temperature, frozen overnight, thawed and dried in an oven at 60 °C for 24 h. The agar loss was calculated as percent loss on a dry weigh basis (%db) as

$$\text{Agar loss (\%db)} = [(\text{Pre-treated agar dry weight (g)} - \text{NaOH dry weight (g)}) / \text{Seaweed Dry weight (g)}] \times 100$$

8.3. RESULTS

8.3.1. Agar yield

The agar yield from different stages of *G. cliftonii* with the all alkali concentrations with or without heating (both groups) are shown in Figure 8.1 A and B. In the first group (alkali treatment without heating), native agar yield of the tetrasporophyte stage was significantly higher ($p < 0.05$) than that of the vegetative stage. The native agar yield for all life stages reduced significantly ($p < 0.01$) with alkali treatment. For alkali treated samples with 0.3 % alkali, agar yield of the vegetative stage was significantly higher ($p < 0.05$) than the carposporophyte and tetrasporophyte stages. However, for samples treated with 0.5 % alkali, agar yield of the tetrasporophyte stage was significantly higher ($p < 0.05$) than the vegetative stage. Agar yield from the vegetative stage treated with a 0.3 % alkali concentration was significantly higher ($p < 0.05$) than 0.5 % alkali.

In the second group (with 1 h heating), native agar yield (control) of the carposporophyte and tetrasporophyte stages was significantly higher ($p < 0.01$) than the vegetative stage. In addition, native agar yield of the carposporophyte and

tetrasporophyte stages were significantly higher ($p<0.05$) than that with alkali treatment while for the vegetative stage it was significantly lower ($p<0.05$) than with alkali treatment. Agar yield from 0.3 and 0.5 % alkali treated vegetative stage were significantly higher ($p<0.01$) than the carposporophyte and tetrasporophyte stages at same alkali concentration.

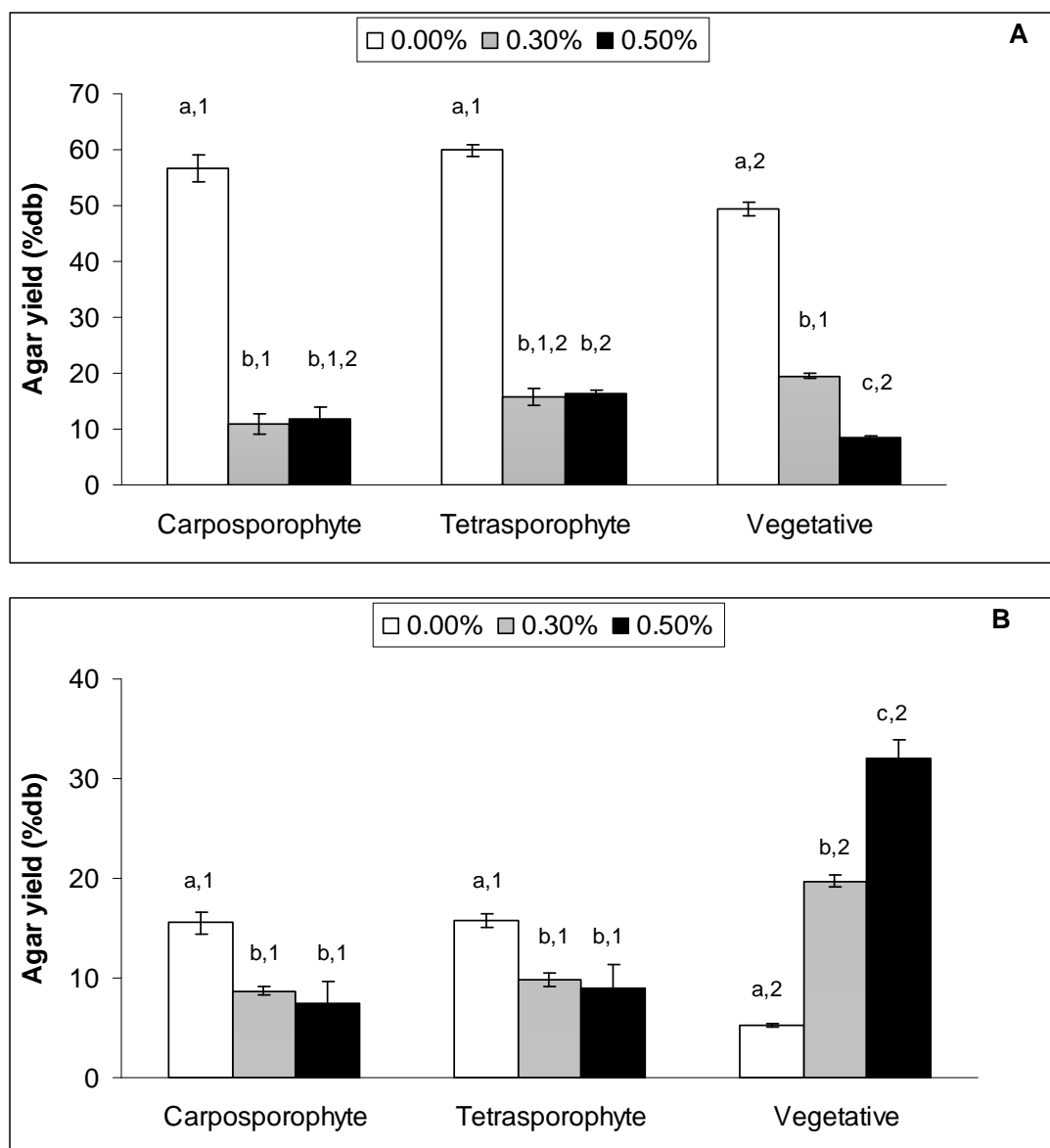


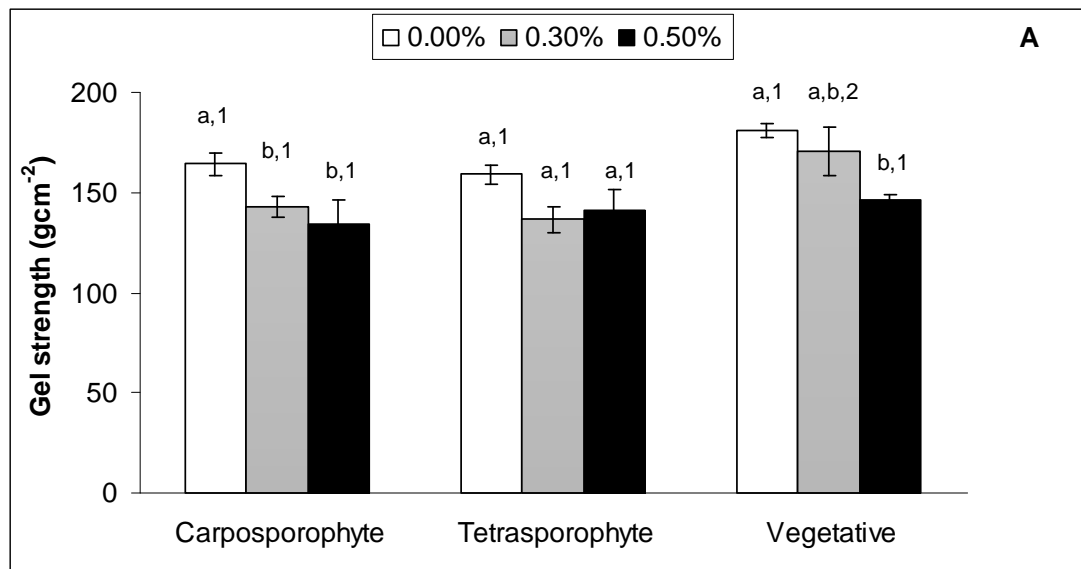
Figure 8.1: Mean agar yield (%db) from *Gracilaria cliftonii* life stages treated with 0.0, 0.3 and 0.5 % alkali concentration (A) without heating and (B) with 1 h heating time

*Error bars represent standard error of mean. Letters a, b, c represent significant difference between alkali concentrations while numbers 1, 2, 3 represents significant differences between stages

8.3.2. Gel strength

The gel strength of agar extracted with different alkali concentrations with or without heating for *G. cliftonii* life stages are shown in Figure 8.2 A and B. In the first group (without heating), gel strength of native agar from all life stages of *G. cliftonii* was not significantly different between life stages. Alkali treatment significantly reduced ($p < 0.05$) the gel strength of agar from carposporophyte as compared to native agar. In addition, native agar gel strength from vegetative was higher ($p < 0.05$) than 0.5 %alkali treatment. Gel strength of agar with 0.3 % alkali treated vegetative stage was significantly higher ($p < 0.05$) than carposporophyte and tetrasporophyte stages at same alkali concentration.

In second group (with 1 h heating), gel strength of native agar from vegetative stage was significantly higher ($p < 0.05$) than carposporophyte but with 0.3 and 0.5 % alkali treatment, gel strength of agar from all life stages of *G. cliftonii* was not significantly different ($p > 0.05$) between each other. Alkali treatment of carposporophyte significantly increased ($p < 0.05$) the agar gel strength as compared to native agar.



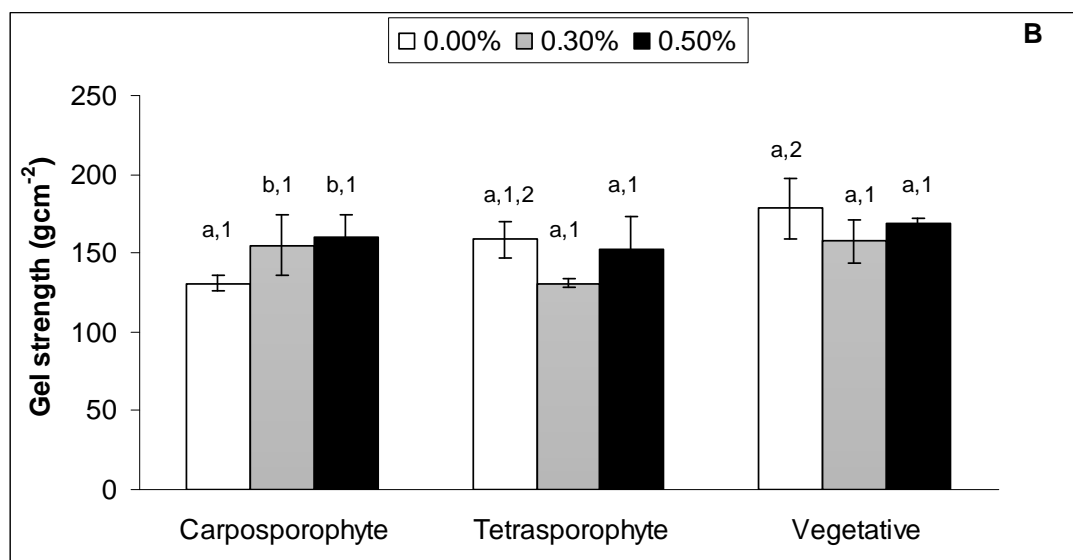


Figure 8.2: Mean gel strength (g.cm^{-2}) of agar from *Gracilaria cliftonii* life stages treated with 0.0, 0.3 and 0.5 % alkali concentration (A) without heating and (B) with 1 h heating time.

*Error bars represent standard error of mean. Letters a, b, c represent difference between alkali concentrations while numbers 1, 2, 3 represents significant differences between stages

8.3.3. Gelling temperature

The effect of alkali concentrations on the gelling temperature of agar extracted from *G. cliftonii* life stages for both groups (with or without heating) is shown in Figure 8.3 A and B. In first group (without heating), gelling temperature of native agar from carposporophyte and vegetative was significantly higher ($p < 0.05$) than from tetrasporophyte stage. However, there was no significant difference in gelling temperatures of agar from all the stages with alkali treatment. The gelling temperature of native agar from vegetative stage was significantly higher ($p < 0.05$) than from 0.5 % alkali treated. In second group (with 1 h heating), gelling temperature of native agar from different life stages and with alkali treatment showed no significant difference ($p > 0.05$) with each other.

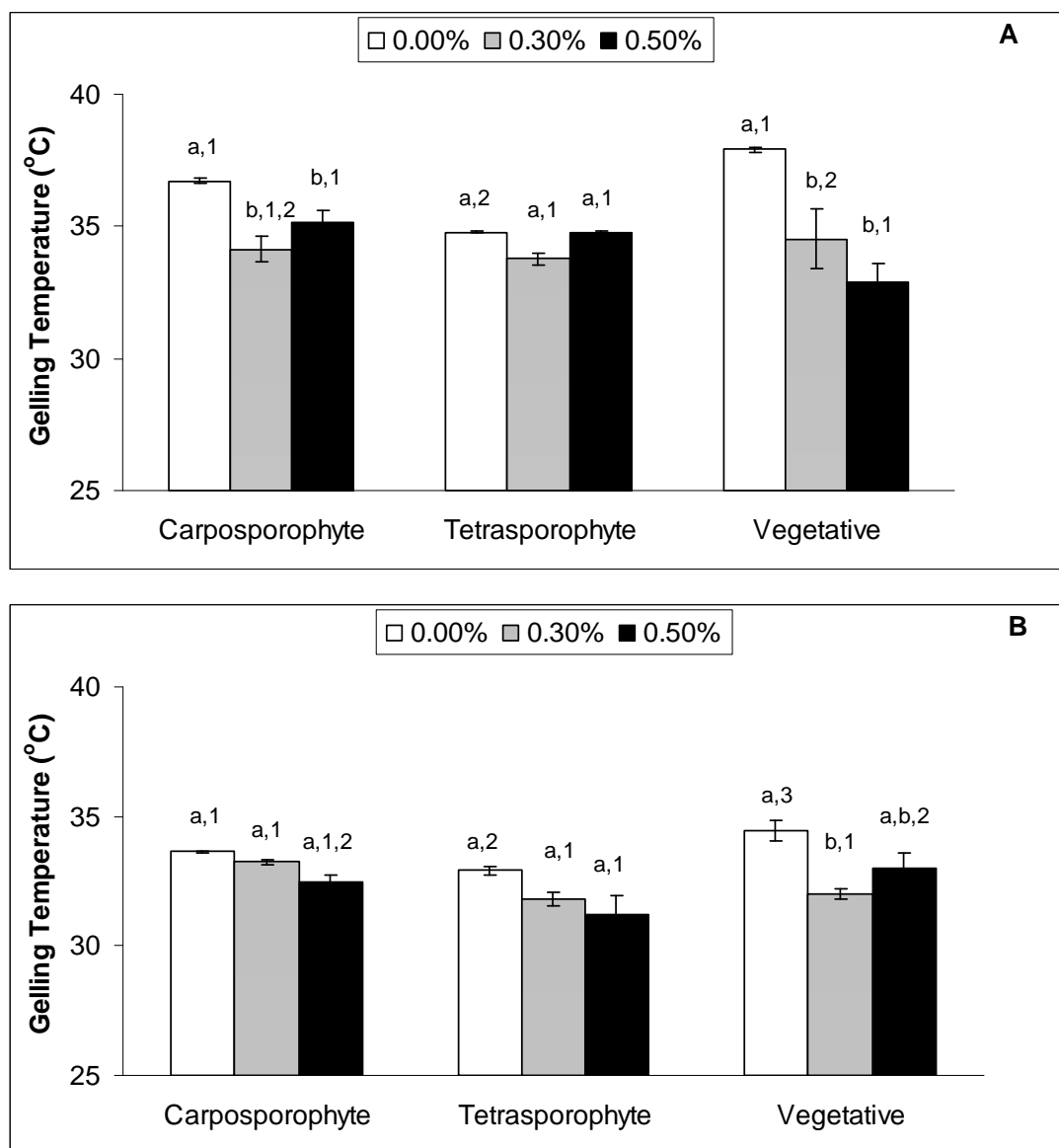


Figure 8.3: Mean gelling temperature (°C) of agar from *Gracilaria cliftonii* life stages treated with 0.0, 0.3 and 0.5 % alkali concentration (A) without heating and (B) with 1 h heating time.

*Error bars represent standard error of mean. Letters a, b, c represent difference between alkali concentrations while numbers 1, 2, 3 represents significant differences between stages

8.3.4. Melting point

The melting point of agar extracted with different alkali concentrations and from different life stages of *G. cliftonii* for both groups (with or without heating) is shown in Figure 8.4 A and B. In first group (without heating), melting point of native agar from carposporophyte, tetrasporophyte and vegetative stages were significantly different ($p < 0.05$) to each other but no significant differences were observed between different stages with alkali treatment. Carposporophyte and tetrasporophyte stages

showed significant differences ($p < 0.05$) between melting point of native agar and alkali treatment. In second group (with 1 h heating), melting point of agar from all life stages with or without alkali treatment were significantly different ($p < 0.05$) to each other. The melting point of native agar of carposporophyte stage was significantly higher than with 0.5 % alkali treatment while for vegetative stage, melting point of native agar was significantly higher ($p < 0.05$) than with 0.3 % alkali treatment. Melting point of agar from tetrasporophyte stage with or without alkali treatments were significantly different to each other ($p < 0.05$).

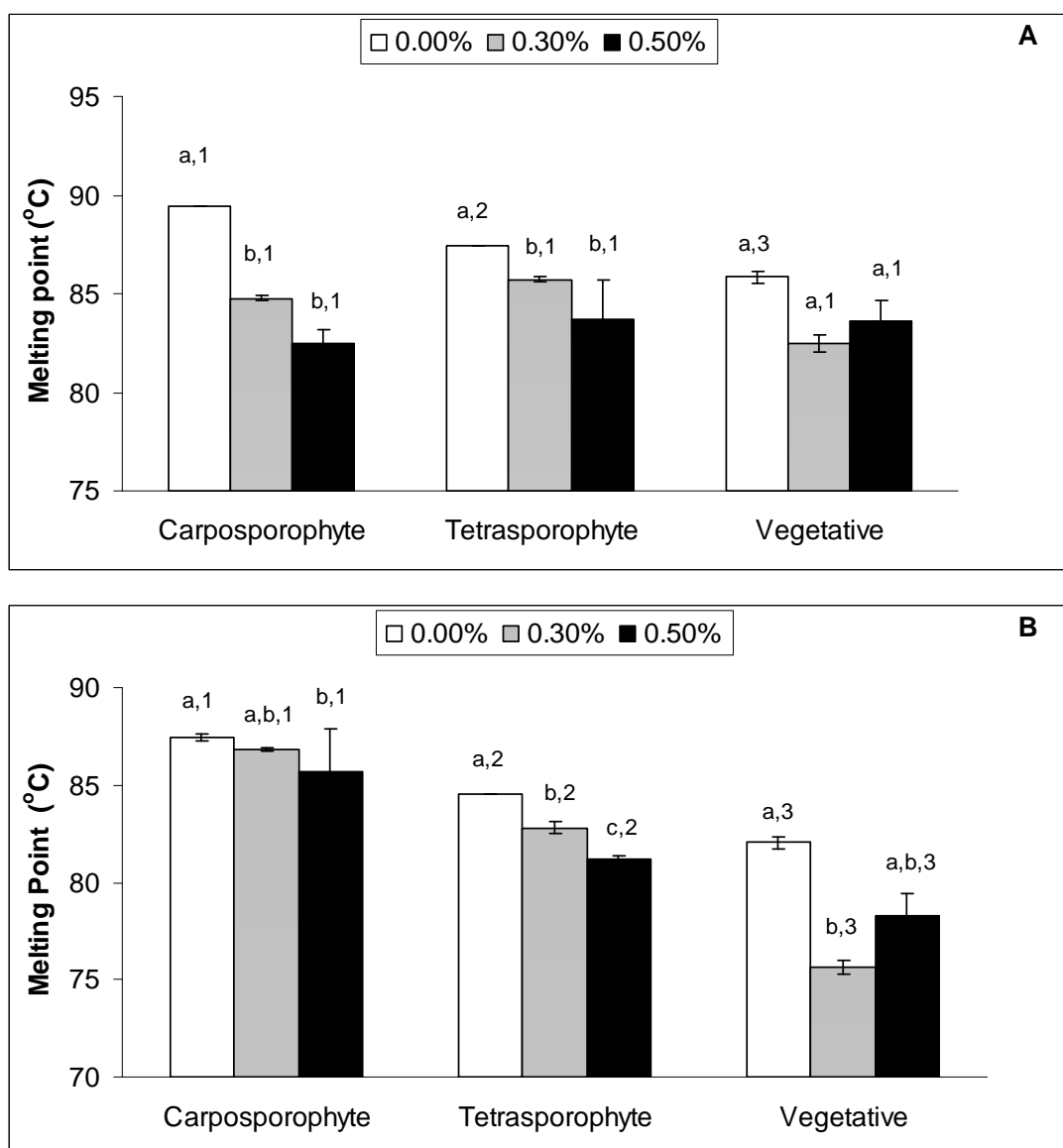


Figure 8.4: Mean melting point ($^{\circ}\text{C}$) of agar from *Gracilaria cliftonii* life stages treated with 0.0, 0.3 and 0.5 % alkali concentration (A) without heating and (B) with 1 h heating time.

*Error bars represent standard error of mean. Letters a, b, c represent difference between alkali concentrations while numbers 1, 2, 3 represents significant differences between stages

8.3.5. Sulphate content

The sulphate content of agar from different stages treated with different alkali concentrations for both groups (with or without heating) is shown in Figure 8.5 A and B. In first group (without heating), sulphate content of native agar and with 0.5 % alkali treatment from different stages was significantly different ($p>0.05$) to each other with maximum sulphate content for vegetative stage. The sulphate content of agar from 0.3 % alkali treated vegetative stage was significantly higher ($p<0.001$) than other two stages at same alkali concentration. Alkali treatment of tetrasporophyte stage had no influence on sulphate content of agar. However, alkali treatment of vegetative and carposporophyte stage resulted in significant decrease ($p<0.05$) of sulphate content of agar as compared to native agar. In second group (with 1 h heating), sulphate content of native agar from different stages was significantly different ($p>0.05$) to each other with maximum for vegetative stage. Sulphate content of agar from alkali treated vegetative stage was significantly higher ($p<0.05$) than the reproductive stages. Alkali treatment of tetrasporophyte stage had no influence on sulphate content of agar but 0.5 % alkali treatment of vegetative and carposporophyte stage significantly decreased ($p<0.05$) the sulphate content of agar as compared to native agar .

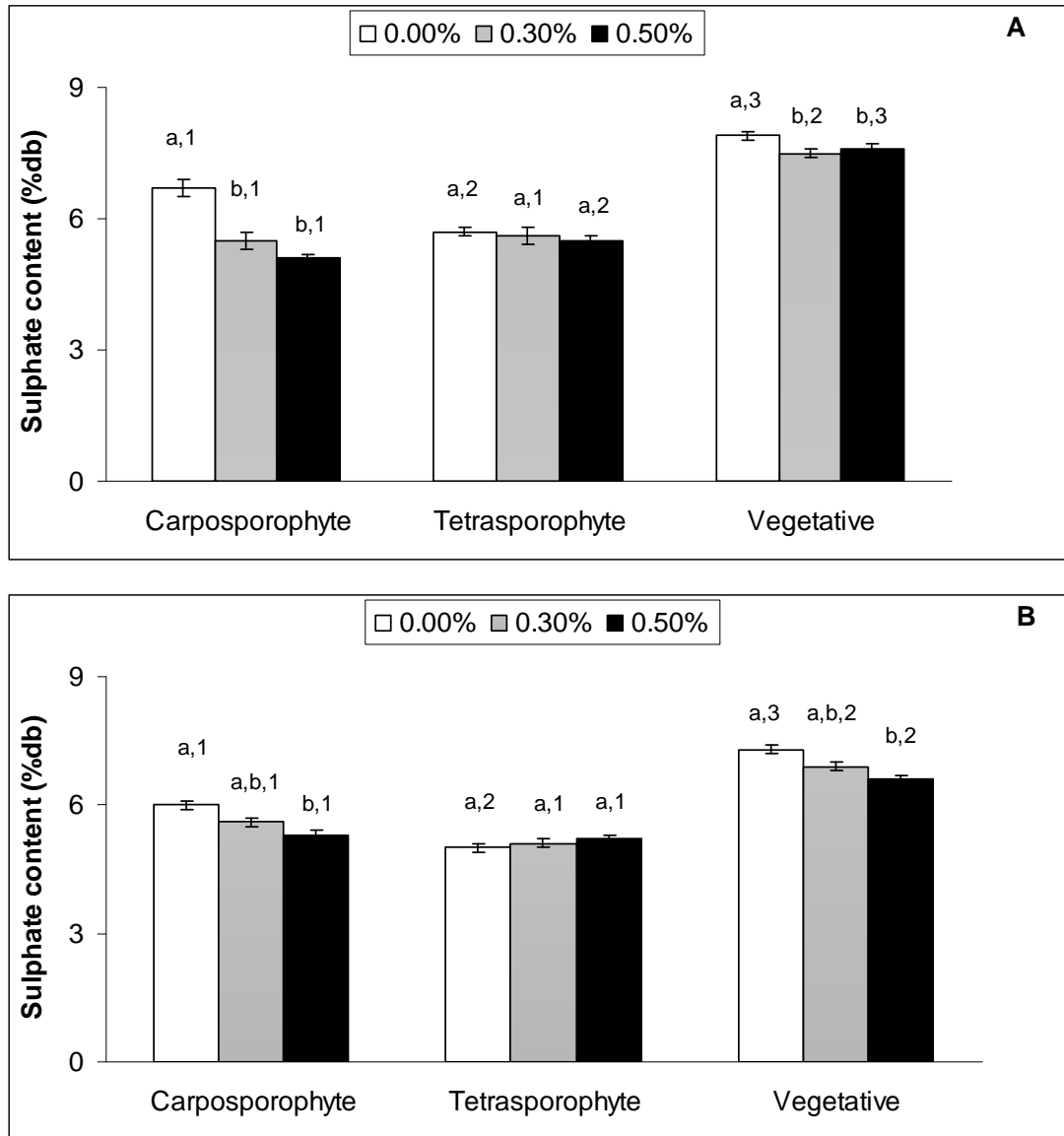


Figure 8.5: Mean sulphate content (%db) of agar from *Gracilaria cliftonii* life stages treated with 0.0, 0.3 and 0.5 % alkali concentration (A) without heating and (B) with 1 h heating time

*Error bars represent standard error of mean. Letters a, b, c represent difference between alkali concentrations while numbers 1, 2, 3 represents significant differences between stages

8.3.6. Agar loss

The agar loss due to alkali treatment of different life stages with 1 h heating time is shown in Figure 8.6. Agar loss from 0.5 % alkali treated tetrasporophyte stage was significantly higher ($p < 0.05$) than other two stages. The agar loss without alkali treatment from vegetative stage was significantly higher ($p < 0.05$) than that from 0.5 % alkali treated.

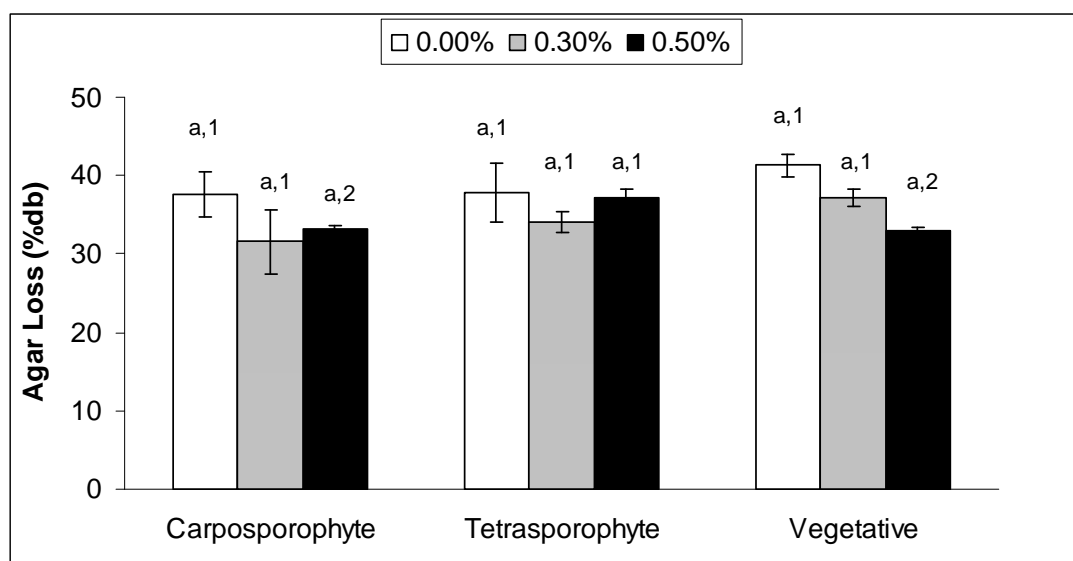


Figure 8.6: Mean agar loss (%db) due to alkali treatment of *Gracilaria cliftonii* life stages treated with 0.0, 0.3 and 0.5 % alkali concentration with 1 h heating time.

*Error bars represent standard error of mean. Letters a, b, c represent difference between alkali concentrations while numbers 1, 2, 3 represents significant differences between stages

8.4. DISCUSSION

Native agar properties from different life stages of *G. cliftonii* and influence of alkali treatment on agar properties have not been reported. This experiment showed that the yield, gel strength, gelling temperature, melting point and sulphate content of agar are life stage dependant for *G. cliftonii* and are influenced by alkali treatment.

In present study, native agar yield of carpoporophyte and tetrasporophyte stages of *G. cliftonii* was higher than 52 %db as reported by Byrne *et al.* (2002). The difference in the yields could be due to the difference in extraction process and stages. However, same author did not specify the stages of *G. cliftonii* used for extraction. The yields of native agar from all the stages of *G. cliftonii* are higher than agar yield from *G. bursapastoris*, *G. coronopifolia* and *G. verrucosa* life stages (Hoyle, 1978; Marinho-Soriano *et al.*, 1999; Penniman, 1977; Whyte *et al.*, 1981) as well as other *Gracilaria* species (Doty *et al.*, 1983; Duckworth *et al.*, 1971; Falshaw *et al.*, 1999; Lawson, 1954; Marinho-Soriano, 2001; Mohamed & Halim, 1952; Santos & Doty, 1983). The high yield of tetrasporophyte stage can be related to its structural difference to vegetative stage as former being reproductive (Kling & Bodard, 1987; Penniman, 1977; Whyte, 1971). The present study suggests that the

lower agar quality observed in carposporophyte and tetrasporophyte plants is due to the great energy required for reproduction. During intensive reproduction, some energy is diverted from agar biosynthesis to reproduction (Marinho-Soriano *et al.*, 1999).

Decrease of *Gracilaria* agar yield with alkali treatment is well known (Buriyo & Kivaisi, 2003). Armisen (1987) also suggested that alkali treatment of *Gracilaria* should be carried out carefully to avoid agar loss which during the process. This could be due to the degradation of polysaccharides by alkali or agar diffusion during the processing, as shown on *G. crassissima* by Freile-Peleguin and Murano (2005). The higher agar yield from vegetative stage when treated with 0.3 %alkali than other stages can be due to the changes in agar structure caused by exposure to alkali (Marinho-Soriano *et al.*, 1999). Similar explanation can be given for high yield of 0.5 %alkali treated tetrasporophyte stage compared to vegetative stage. Marinho-Soriano, *et al.* (1999) suggested that the structural differences between different life stages responsible for differences in agar yield in *G. bursapastoris*. Armisen (1987) suggested that alkali treatment is dependent on variables like temperature and alkali concentrations and varies among *Gracilaria* species. In present study, alkali treatment with 1 h heating at 70 °C resulted in considerable agar loss. This is due to the fact that agar was extracted during the alkali treatment itself indicating that temperature of 70 °C is too high for alkali treatment. In addition, the loss in agar suggest that *G. cliftonii* contain important quantities of alkali-labile sulphates which might have been lost during the process (Buriyo & Kivaisi, 2003).

Gel strength from native agar showed differences between different life stages of *G. cliftonii*. The carposporophyte stage presented low gel strength (164.2 g.cm⁻²) than vegetative stage (180.8 g.cm⁻²). These results are in agreement with those published by Marinho-Soriano, *et al.* (1999) who found that the gel strength of the agar from carposporophyte stage was weaker than that from vegetative stage but are contradiction to those reported by Penniman (1977), Hoyle (1978) and Whyte *et al.* (1981). Low gel strength of agar from carposporophyte and tetrasporophyte stages could be due to their high energy requirements for reproduction being not able to synthesize high quality agar (Marinho-Soriano *et al.*, 1999). However, gel strength of native agar from *G. cliftonii* is higher than that reported for *G. bursapastoris* (Marinho-Soriano *et al.*, 1999) and *G. coronopifolia* (Hoyle, 1978). The adverse effect of alkali treatment was evident on gel strength as it decreased with increase in

alkali concentrations. This can be due to various factors for example, gel strength of *Gracilaria* agars are dependent on (i) species (Cote & Hanisak, 1986) (ii) environmental factors (Craigie & Wen, 1984) (iii) seasonal variations (Lahaye & Yaphe, 1988) and (iv) extraction methods (Armisen & Galatas, 1987). In addition, gel strength is reported to be affected by the instrument used and operating parameters (like speed of plunger, container, plunger diameter, curing of gel) which in the present study is different to those reported in literature (Chapter 1).

Several investigations have demonstrated that depending on sulphate position, the gelling mechanism of agar can be inhibited or delayed (Andriamanananatony *et al.*, 2007; Craigie & Jurgens, 1989; Duckworth & Yaphe, 1971; Goncalves *et al.*, 2006; Lahaye & Rochas, 1991; Usov, 1998). The higher sulphate content of agar from the vegetative stages in both groups are in confirmation with studies on *G. bursapastoris* by Marinho-Soriano, *et al.*, (1999) and Whyte *et al.* (1981). Sulphate content reduced significantly with alkali treatment in both the groups and showed negative correlation with gel strength for carposporophyte stage. The possible reason can be alteration in position of sulphate in the agar structure or conversion of L-galactose-6-sulphate to 3,6-anhydro-L-galactose (Andriamanananatony *et al.*, 2007; Craigie & Jurgens, 1989; Duckworth *et al.*, 1971; Freile-Pelegrin & Murano, 2005; Freile-Pelegrin & Robledo, 1997b; Orduña-Rojas *et al.*, 2008). In addition, the reduction in sulphate content of agar in second group could be due to the loss of alkali-labile sulphates while processing (Buriyo & Kivaisi, 2003).

Gelling temperature, melting point and gel strength are indicative of agar quality. These characteristics are closely dependent on the chemical structure of the polysaccharide (Marinho-Soriano *et al.*, 1999). The agar extracted from the carposporophyte and vegetative stages showed gelling temperatures higher than that from the tetrasporophyte stage. Marinho-Soriano, *et al.*, (1999) and Whyte *et al.* (1981) studied this property in the life stages of *G. bursapastoris* and *G. gracilis* respectively and showed differences between different stages but no explanation was provided. The present results are in agreement for carposporophyte stage but are contradictory for tetrasporophyte and vegetative stage. The variation in gelling temperature of agar could be due to the change in methoxyl content and its location in chemical structure along with change in molecular weight distribution (Andriamanananatony *et al.*, 2007; Guiseley, 1970; Rebello *et al.*, 1997). Melting point of agar from vegetative stage was significantly lower than reproductive stages.

The differences in melting point could be due to the difference in the internal structure of different stages (Marinho-Soriano *et al.*, 1999) and the extraction process (Armisen & Galatas, 1987). In addition, the difference in melting point could be due to change in the pyruvic acid content of agar (Young *et al.*, 1971). However, the melting point and gelling temperature of native agar from different life stages was in the range specified by US Pharmacopeia (82-87 °C and 32-37 °C respectively) for agar usage.

In conclusion, the analysis of agar extracted from the different stages of *G. cliftonii* showed the influence of the biological cycle of the seaweed on the quality of agar confirming the work of Penniman (1977), Whyte *et al.* (1981) and Marinho-Soriano, *et al.*, (1999). The alkali treatment used in present study had adverse affect on agar yield and quality from different stages of *G. cliftonii*. In addition, alkali treatment with heating resulted in agar loss. However, reduction in sulphate content due to alkali treatment is positive indication and agar quality should be tested with alkali treatments in conjunction with other variables like heating and soaking.

**CHAPTER 9: MODIFICATION OF ALKALI TREATMENT - LOW
ALKALI CONCENTRATION, SOAKING TIME AND HEATING
TIME**

9.1. INTRODUCTION

In Chapter 8, alkali treatment prior to extraction of agar and heating for 1 h during agar extraction resulted in reduction of agar yield and quality from *G. cliftonii*. Concentration of alkali, heating and soaking time of the alkali treatment for *Gracilaria* are reported to affect the yield and quality of agar (Andriamanananatonic et al., 2007; Armisen & Galatas, 1987; Arvizu-Higuera et al., 2008; Li et al., 2008; Orduña-Rojas et al., 2008). As discussed in Chapter 8, *Gracilaria* species produce low quality agars due to high sulphate concentrations (Armisen & Galatas, 1987). It has been reported that the gel properties of many *Gracilaria* agars can be improved by adding an alkali treatment before extracting agar to convert L-galactose-6-sulphate to 3,6-anhydro-L-galactose, which is responsible for the enhancement of the gel forming ability (Duckworth et al., 1971; Freile-Pelegrin & Murano, 2005; Freile-Pelegrin & Robledo, 1997b; Orduña-Rojas et al., 2008). In addition, the alkali treatment of *Gracilaria* species must be adapted for each species and variables such as concentration, heating time and temperature must be adjusted to obtain as much desulphation as possible and to avoid yield losses (Armisen & Galatas, 1987; Freile-Pelegrin & Robledo, 1997b; Orduña-Rojas et al., 2008).

The purpose of soaking in water is to hydrate the seaweeds and ease the availability of soluble polysaccharides in seaweeds. Although, different soaking times are used for seaweeds prior to gel extraction, the effect of soaking time has not been reported for any *Gracilaria* species (Chapter 1). The main constituents of seaweeds are hydrophilic polysaccharides and water reacts with both surface (external) and internal polysaccharides by hydrogen bonding.

The present study was conducted to investigate the effect of low alkali concentration, soaking and heating time and their interaction with alkali treatment of *G. cliftonii* on the agar quality characteristics

9.2. MATERIAL AND METHODS

9.2.1. Sample and alkali preparation

G. cliftonii fronds were washed with tap water to remove sand, mud and

epiphytes. The 'clean seaweed' was oven dried for 8 h at 60 °C and stored in separate sealed plastic bags until agar extraction. Three concentrations 0.3, 0.5 and 1 % alkali were prepared by dissolving analytical grade sodium hydroxide in distilled water.

9.2.2. Alkali treatment

To modify alkali treatment of *G. cliftonii* samples were divided into two equal groups. In the first group, samples of 5 g dry weight each were soaked in 0.3, 0.5 and 1.0 % alkali for 0, 1, 2 and 3 h in 200 mL of respective alkali solution at room temperature (25 °C) followed by heating time of 1 h in a waterbath at 70 °C in triplicates. Samples without soaking (soaking time = 0 h) were hydrated for 1-2 minutes in 200 mL of respective alkali solution at room temperature. The samples without alkali (0.0 % alkali) were soaked in distilled water for 1, 2 and 3 h, followed by heating time of 1 h in a water bath at 70 °C in triplicates. The samples without alkali (0.0% alkali) and soaking time (soaking time = 0 h) followed by heating time of 1 h in a water bath at 70 °C were used as the control in triplicates. In the second group, the same steps were followed but with a heating time of 2 h in a water bath at 70 °C.

After alkali treatment, the solution was transferred into plastic containers to determine agar loss due to treatment as

Agar loss = [(pre-treated agar dry weight (g)-Weight of NaOH (g)/Seaweed dry weight (g))] x 100

9.2.3. Agar extraction

Alkali removal and agar extraction was performed using the methodology described in Chapter 8. Modification of the alkali treatment and agar extraction process is shown in Appendix 4.

9.3. RESULTS

Alkali treatment concentration with different soaking times and heating time of 1 and 2 h influenced the agar characteristics of *G. cliftonii*. Overall the results show that alkali treatment of *G. cliftonii* reduced the agar yield significantly ($p < 0.01$) and resulted in more than 30 % agar loss.

9.3.1. Alkali treatment with 1 h heating time (Group 1)

Agar yield of *G. cliftonii* treated with low alkali concentrations and 1-3 h soaking times was in range 12.4-31.8 %db (Figure 9.1). Alkali treatment significantly reduced ($p<0.01$) the agar yield. In addition, agar yield after 0.3 % alkali treatment was significantly higher ($p<0.05$) than after 1.0 % alkali treatment with soaking times of 1 and 3 h. The agar yield from samples without alkali treatment and soaking time was significantly higher ($p<0.05$) than samples with alkali treatment and soaking. Soaking times between 1-3 h did not influence the agar yield for samples without alkali treatment. However, agar yield of *G. cliftonii* with soaking time of 2 h was significantly lower ($p<0.01$) than without soaking and 1 h soaking treated with 0.3 % alkali. For sample treatment with 0.5 and 1.0 % alkali at different soaking times, agar yield was significantly reduced ($p<0.01$) as compared to samples without soaking.

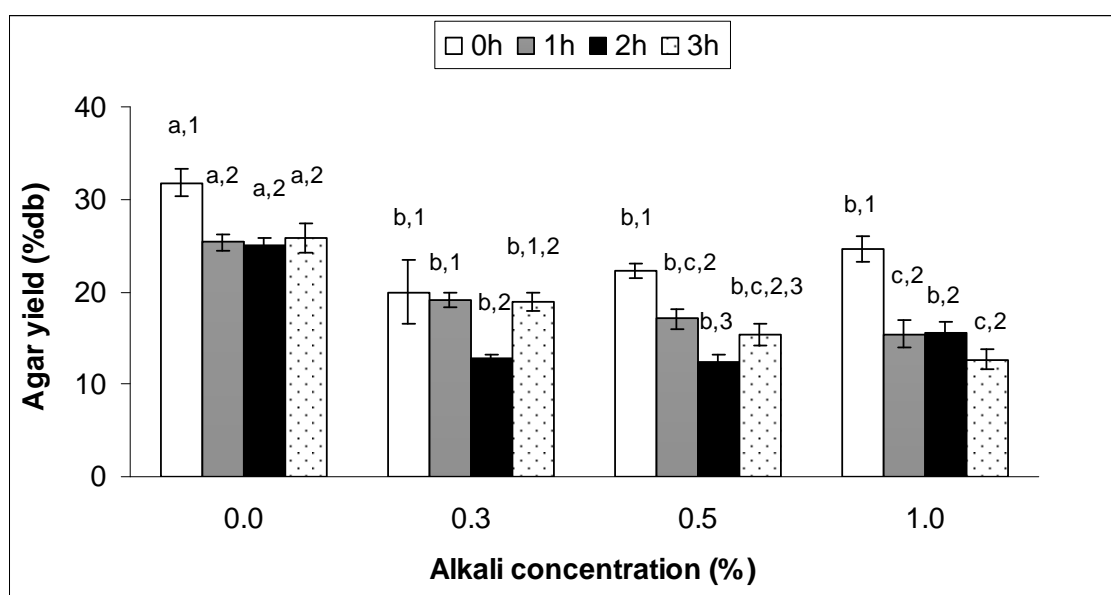


Figure 9.1: Mean agar yield (%db) from *Gracilaria cliftonii* treated at different alkali concentrations with soaking times of 0, 1, 2 and 3 h

*Error bars represent standard error of mean. Letters a, b, c represent difference between alkali concentrations while numbers 1, 2, 3 represents significant differences between soaking times

Agar loss was observed with or without alkali treatment with different soaking times (Figure 9.2). Agar loss from *G. cliftonii* without alkali treatment was significantly lower ($p<0.01$) than with alkali treatment for different soaking times except for 3 h soaked samples. Agar loss for the control and 1.0 % alkali treated

samples without soaking was significantly lower ($p < 0.05$) than soaked samples. Different soaking times did not affect the agar loss after 0.3 and 0.5 % alkali treatment.

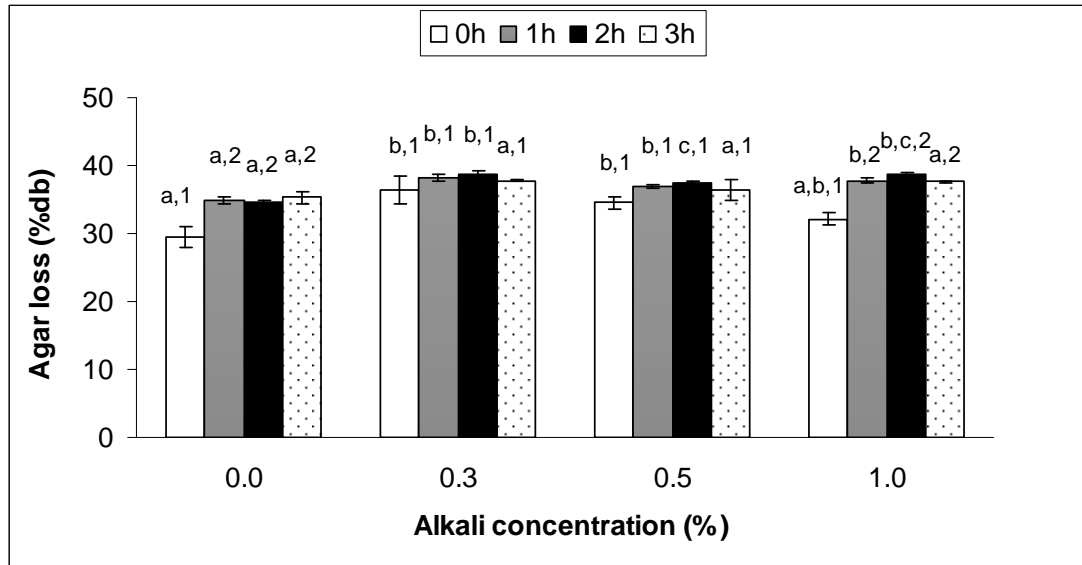


Figure 9.2: Mean agar loss (%db) from *Gracilaria cliftonii* treated at different alkali concentrations with soaking times of 0, 1, 2 and 3 h

*Error bars represent standard error of mean. Letters a, b, c represent difference between alkali concentrations while numbers 1, 2, 3 represents significant differences between soaking times

Gel strength of agar with or without alkali treatment with different soaking times was in the range of 107.1-178.3 g.cm⁻² (Figure 9.3). Gel strength of agar for 1 h soaked *G. cliftonii* with 0.3 % alkali treatment was significantly ($p < 0.05$) higher than with 1.0 % alkali treatment. Gel strength of agar from 2 h soaked samples without alkali treatment was significantly higher ($p < 0.05$) than with 1.0 % alkali treatment. Gel strength of agar of samples with or without alkali treatment was maximum when soaked for 2 h and was significantly higher ($p < 0.05$) than 1 and 3 h soaked samples. In addition, gel strength of agar for samples treated with 0.5 % alkali and soaked for 3 h was significantly higher ($p < 0.05$) than 1 h soaked samples and samples without soaking.

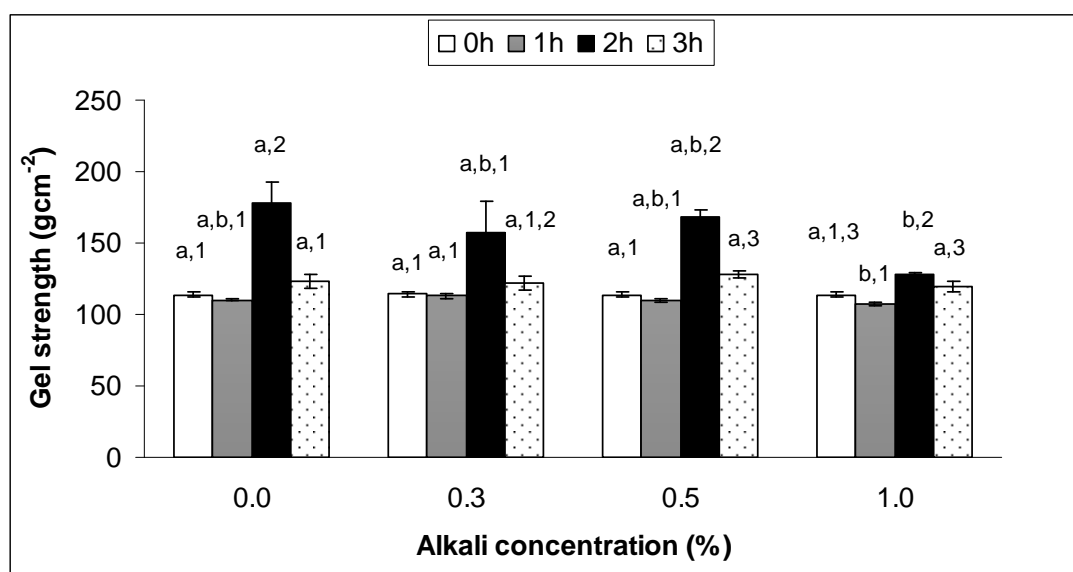


Figure 9.3: Mean gel strength (g.cm^{-2}) of agar from *Gracilaria cliftonii* treated at different alkali concentrations with soaking times of 0, 1, 2 and 3 h

*Error bars represent standard error of mean. Letters a, b, c represent difference between alkali concentrations while numbers 1, 2, 3 represents significant differences between soaking times

Melting point of agar was in the range of 74.9-86.9 °C for different alkali concentrations and soaking times (Figure 9.4). Melting point of agar for 1 h soaked samples treated with 0.3 % alkali was significantly higher ($p < 0.01$) than with 1.0 % alkali but for 2 h soaked samples treated with 0.3 % alkali was significantly lower ($p < 0.01$) than with 1.0 % alkali. In addition, for *G. cliftonii* treated with 0.3, 0.5 and 1.0 % alkali soaking significantly reduced ($p < 0.05$) the melting point of agar.

Gelling temperature of agar for different combinations of alkali concentrations and soaking time are shown in Figure 9.5. Gelling temperature of agar for 2 h soaked samples without alkali treatment was significantly higher ($p < 0.05$) than samples treated with 0.3 and 1.0 % alkali. Other soaking times did not influence the gelling temperature of agar for samples with or without alkali treatment. However, gelling temperature of agar for 1 and 3 h soaked samples without alkali treatment and samples treated with 0.3 and 0.5 % alkali was significantly lower ($p < 0.01$) than samples without soaking. In addition, gelling temperature of agar for samples treated with 1 % alkali without soaking was significantly higher than 2 h soaked samples.

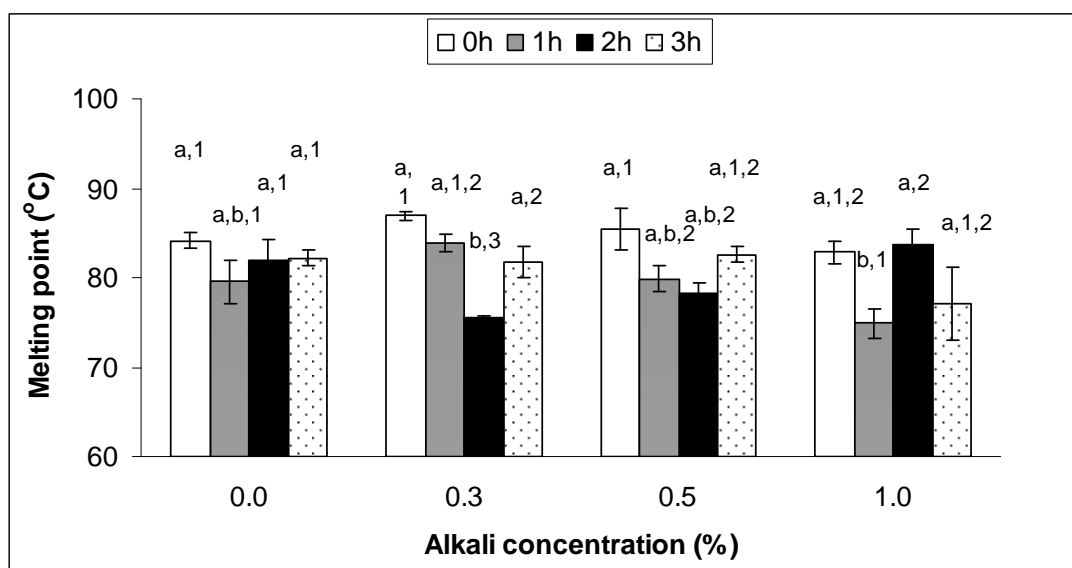


Figure 9.4: Mean melting point (°C) of agar from *Gracilaria cliftonii* treated at different alkali concentrations with soaking times of 0, 1, 2 and 3 h

*Error bars represent standard error of mean. Letters a, b, c represent difference between alkali concentrations while numbers 1, 2, 3 represents significant differences between soaking times

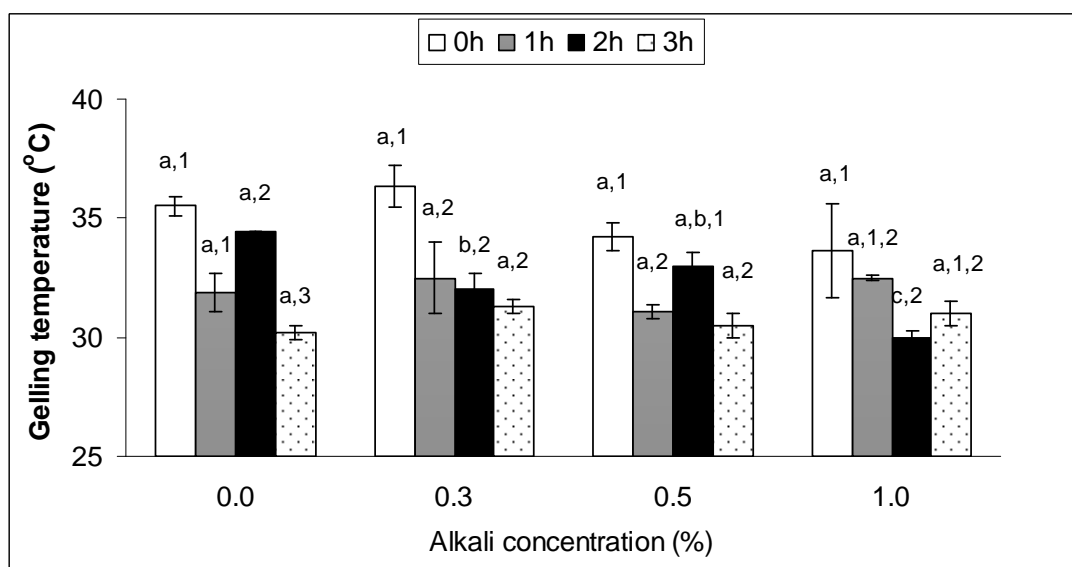


Figure 9.5: Mean gelling temperature (°C) of agar from *Gracilaria cliftonii* treated at different alkali concentrations with soaking times of 0, 1, 2 and 3 h

*Error bars represent standard error of mean. Letters a, b, c represent difference between alkali concentrations while numbers 1, 2, 3 represents significant differences between soaking times

Sulphate content of agar from samples with or without alkali treatment with different soaking times was in range of 5.7-7.3 %db (Figure 9.6). Sulphate content of agar was significantly reduced ($p < 0.05$) for samples treated with 0.5 and 1.0 % alkali

with 0,1 and 2 h soaking times. However, sulphate content of agar for 1 h soaked samples without alkali treatment or treated with 0.3 and 0.5 % alkali was significantly lower ($p<0.05$) than 2 h soaked samples and samples without soaking.

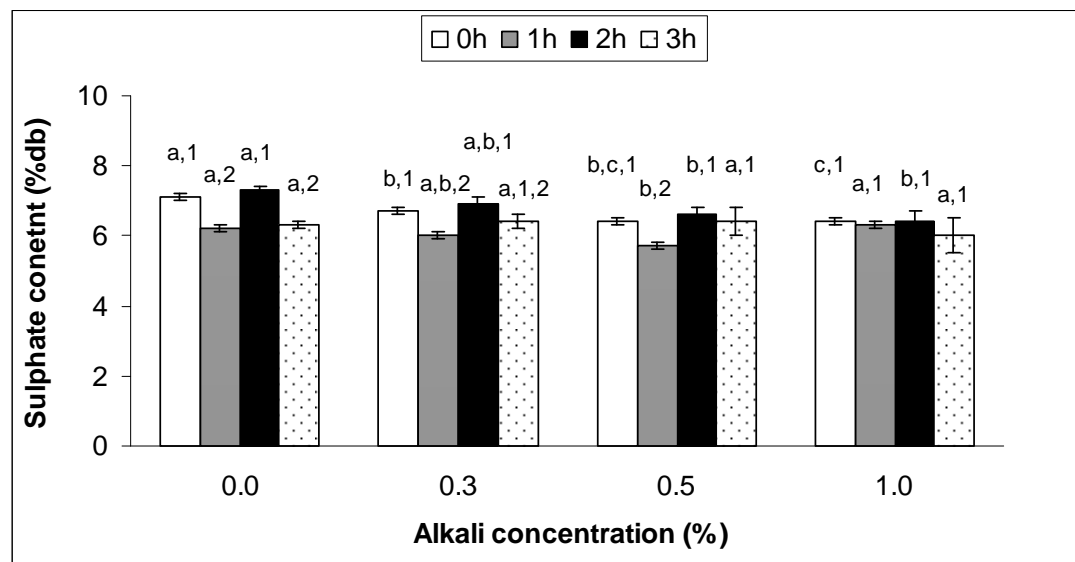


Figure 9.6: Mean sulphate content of agar (%db) from *Gracilaria cliftonii* treated at different alkali concentrations with soaking times of 0, 1, 2 and 3 h

*Error bars represent standard error of mean. Letters a, b, c represent difference between alkali concentrations while numbers 1, 2, 3 represents significant differences between soaking times

9.3.2. Alkali treatment with 2 h heating time (Group 2)

In group 2, the agar yield of *G. cliftonii* samples treated with different alkali concentrations and soaking times was in range of 14.6-28.5 %db (Figure 9.7). Alkali treatment of samples with different soaking time significantly reduced ($p<0.01$) the agar yield. In addition, agar yield from 2 and 3 h soaked samples and treated with 0.3 % alkali was significantly higher ($p<0.05$) than samples treated with 0.5 and 1.0 %alkali. In addition, agar yield from samples treated with 0.3 % alkali with 2 and 3 h soaking times was significantly higher ($p<0.05$) than with 1 h soaked samples and samples without soaking.

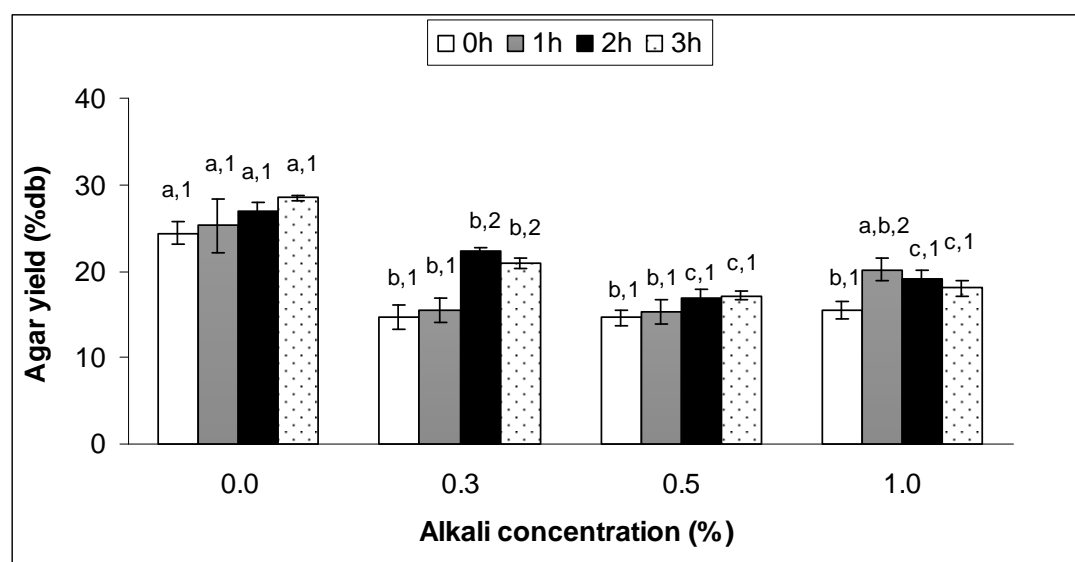


Figure 9.7: Mean agar yield (%db) from *Gracilaria cliftonii* treated for 2 h at different alkali concentration with soaking times of 0, 1, 2 and 3 h

*Error bars represent standard error of mean. Letters a, b, c represent difference between alkali concentrations while numbers 1, 2, 3 represents significant differences between soaking times

Agar loss from *G. cliftonii* samples in group 2, with or without alkali treatment with different soaking times was in the range 32.5 -38.4 %db (Figure 9.8). Agar loss from samples without soaking, samples without alkali treatment and 1.0 % alkali treated was significantly lower ($p < 0.05$) than samples treated with 0.3 and 0.5 % alkali. However, agar loss for 3 h soaked samples without alkali treatment was significantly lower ($p < 0.05$) than with alkali treated samples. Agar loss from samples treated with 0.3 % alkali without soaking was significantly higher ($p < 0.05$) as compared to 2 and 3 h soaked samples. Different soaking times did not affect the agar loss for 0.5 and 1.0 % alkali treated samples.

In group 2, gel strength of agar for *G. cliftonii* samples with or without alkali treatment with different soaking times was in range 107-169 g.cm⁻² (Figure 9.9). Gel strength for 1 and 2 h soaked samples without alkali treatment and with 0.3 % alkali treatment was significantly higher ($p < 0.05$) than with 0.5 and 1.0 % alkali treatment. Gel strength of agar for samples with or without alkali treatment with 1 h soaking time was significantly higher ($p < 0.05$) than other soaking times. Gel strength of agar from samples treated with 0.3 and 0.5 % alkali with 2 and 3 h soaking was significantly higher ($p < 0.05$) than samples without soaking.

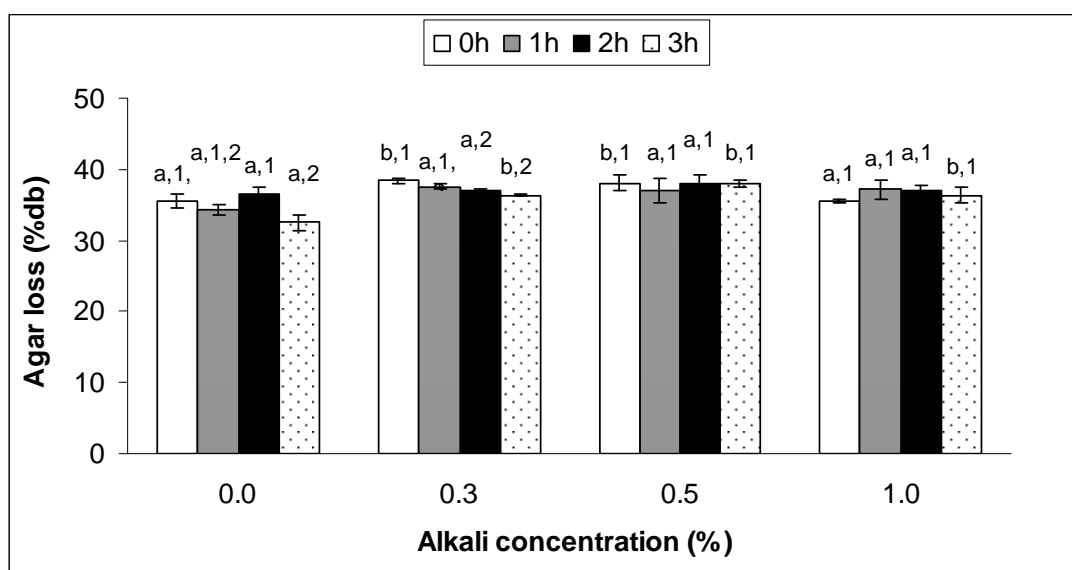


Figure 9.8: Mean agar loss (%db) due to treatment of *Gracilaria cliftonii* for 2 h at different alkali concentration with soaking time of 0, 1, 2 and 3 h

*Error bars represent standard error of mean. Letters a, b, c represent difference between alkali concentrations while numbers 1, 2, 3 represents significant differences between soaking times

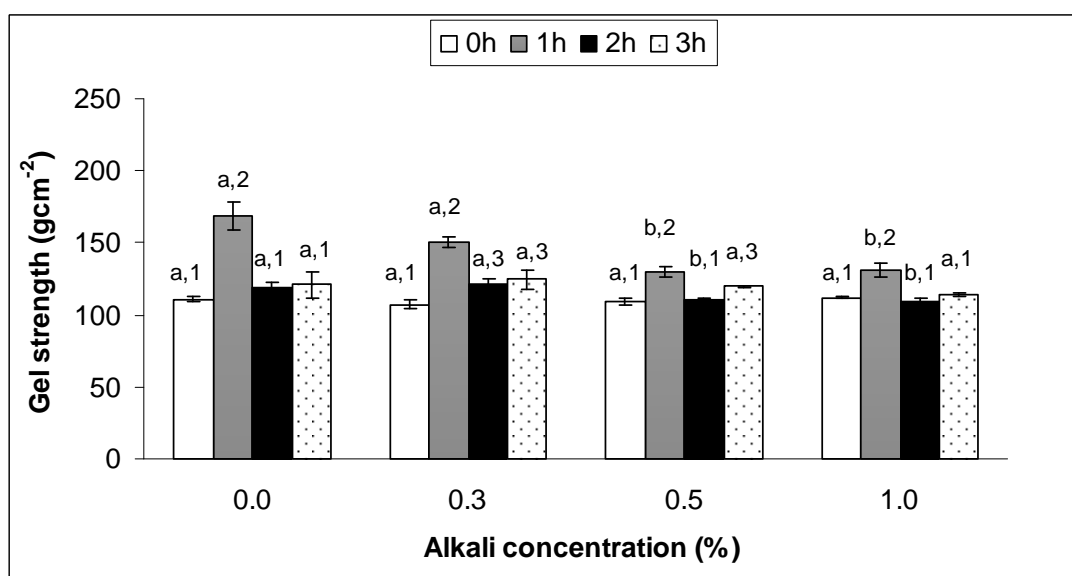


Figure 9.9: Mean gel strength of agar (g.cm⁻²) from *Gracilaria cliftonii* treated for 2 h at different alkali concentration with soaking times of 0, 1, 2 and 3 h

*Error bars represent standard error of mean. Letters a, b, c represent difference between alkali concentrations while numbers 1, 2, 3 represents significant differences between soaking times

Melting point of agar from *G. cliftonii* with different combinations of alkali treatments and soaking times was in the range 72.1-83.9 °C (Figure 9.10). Melting point of agar from 1 h soaked samples without alkali and with 0.3 % alkali treatment

was significantly higher ($p < 0.01$) than with 0.5 and 1.0 % alkali treatment. In addition, the melting point of agar from samples with 0.5 % alkali treatment without soaking and 3 h soaking, was significantly higher ($p < 0.05$) than for 1 and 2 h soaked samples.

Gelling temperature of agar for *G. cliftonii* samples treated with combinations of different alkali concentrations and soaking times was in range 29.3-33.3 °C (Figure 9.11). Gelling temperature of agar significantly reduced ($p < 0.05$) for alkali treated samples with 1 h soaking time. Gelling temperature of agar for 2 and 3 h soaked samples treated without alkali and with 0.3 % alkali was significantly higher ($p < 0.05$) than samples without soaking and with 3 h of soaking time. In addition, the gelling temperature of agar for samples treated with 1.0 %alkali, with 2 h soaking time was significantly higher ($p < 0.05$) than samples without soaking and other soaking times.

Sulphate content of agar for *G. cliftonii* with or without alkali treatment with different soaking times was in range 5-7 %db (Figure 9.12). Sulphate content of agar significantly reduced ($p < 0.05$) with alkali treatment for samples without soaking and with 2 h soaking time. In addition, the sulphate content of agar for samples with and without alkali treatment significantly reduced ($p < 0.05$) with 2 h alkali soaking time as compared to samples without soaking.

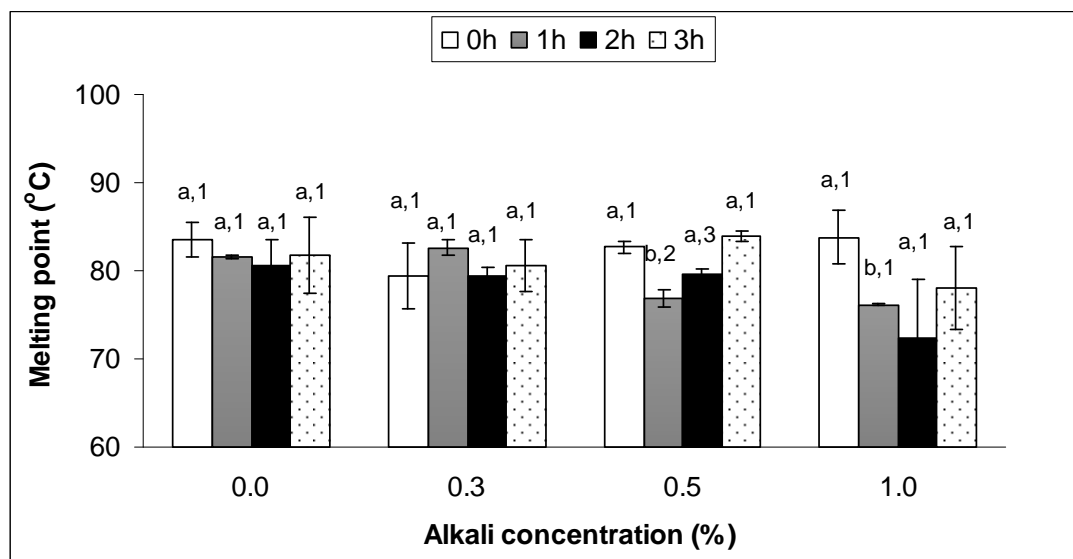


Figure 9.10: Mean melting point of agar (°C) from *Gracilaria cliftonii* treated for 2 h at different alkali concentration with soaking times of 0, 1, 2 and 3 h

*Error bars represent standard error of mean. Letters a, b, c represent difference between alkali concentrations while numbers 1, 2, 3 represents significant differences between soaking times

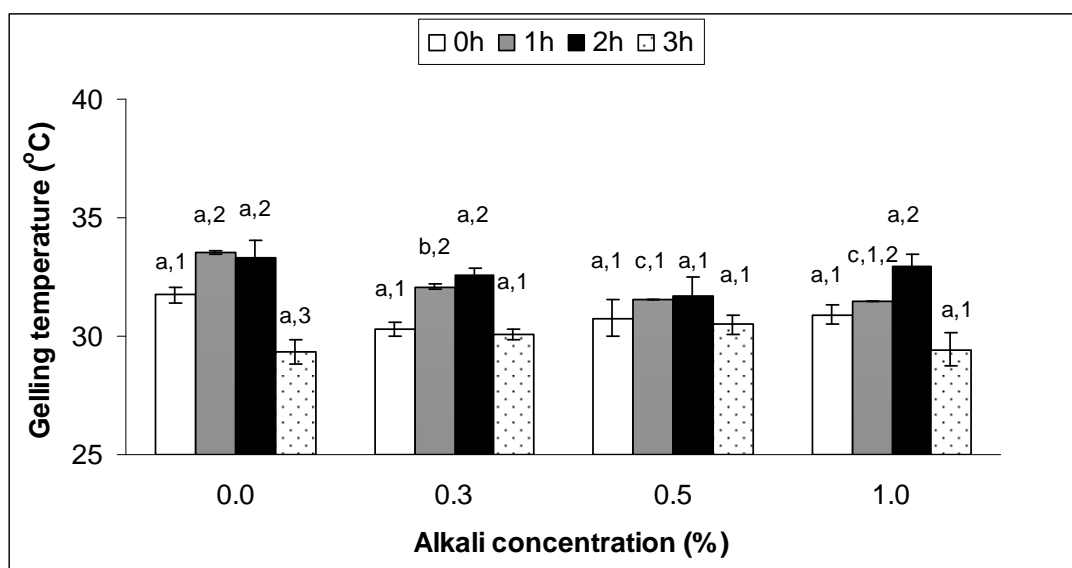


Figure 9.11: Mean gelling temperature of agar (°C) from *Gracilaria cliftonii* treated for 2 h at different alkali concentration with soaking times of 0, 1, 2 and 3 h

*Error bars represent standard error of mean. Letters a, b, c represent difference between alkali concentrations while numbers 1, 2, 3 represents significant differences between soaking times

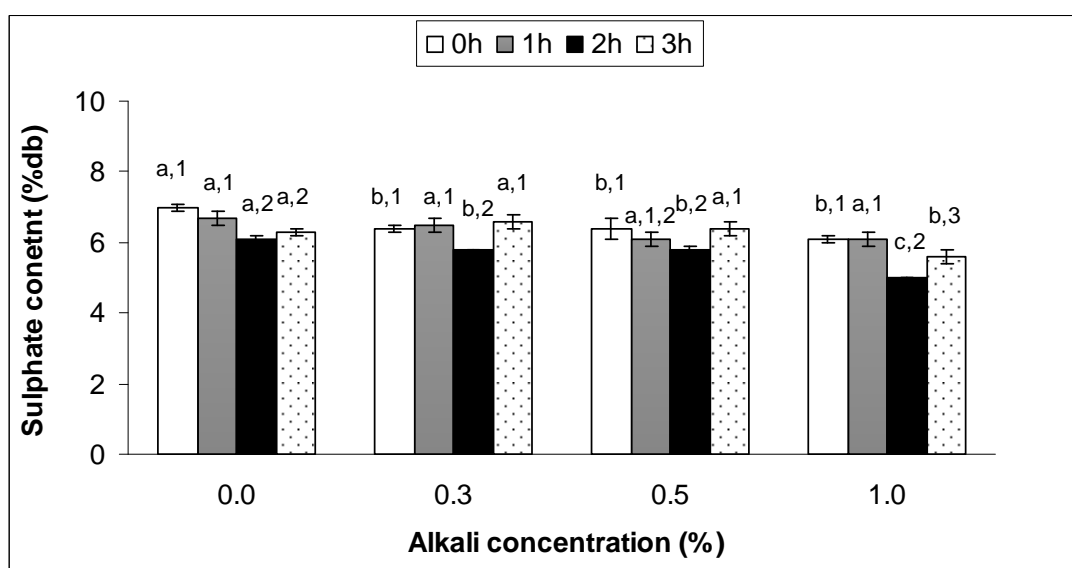


Figure 9.12: Mean sulphate content of agar from *Gracilaria cliftonii* treated for 2 h at different alkali concentration with soaking times of 0, 1, 2 and 3 h

*Error bars represent standard error of mean. Letters a, b, c represent difference between alkali concentrations while numbers 1, 2, 3 represents significant differences between soaking times

9.3.3. Effect of heating time

Agar yield and properties for the samples treated with different alkali concentrations and soaking times show significant differences between different treatment times.

Agar yield from samples without alkali treatment and with 0.5 and 1.0 % alkali treatment, followed by 1 h heating time, without soaking was significantly higher ($p < 0.05$) than with a heating time of 2 h. However, agar yield from 0.3 % alkali treated and 2h soaked samples followed by a heating time of 1 h was significantly lower ($p < 0.05$) than with a heating time of 2 h. Agar loss from samples without alkali treatment and with a soaking time after heating of 1 h was significantly lower ($p < 0.05$) than with a heating time of 2 h. Gel strength of agar for 1 h soaked samples with and without alkali treatment for 1 h heating time was significantly lower ($p < 0.05$) than with a heating time of 2 h. However, gel strength of agar from samples with and without alkali treatment, soaked for 2 h followed by a heating time of 1 h was significantly higher ($p < 0.05$) than with a heating time of 2 h.

Melting point of agar from 0.3 % alkali treated samples soaked for 2 h followed by a heating time of 1 h was significantly higher ($p < 0.05$) than with a heating time of 2 h. However, the melting point of agar from 0.3 % alkali treated samples without soaking followed by a heating time of 1 h was significantly lower ($p < 0.05$) than with a heating time of 2 h. In addition, the melting point for 2 h soaked samples treated with 1.0 % alkali with a heating time of 1 h was significantly lower ($p < 0.05$) than with a heating time of 2 h. Gelling temperature of agar for samples with or without alkali treatment and without soaking time for 2 h heating time was significantly higher ($p < 0.05$) than for 1 h heating time. Sulphate content of agar for 2 h soaked samples with or without alkali treatment for 1 h heating time was significantly lower ($p < 0.05$) than for 2 h heating time.

9.4. DISCUSSION

Alkali concentration and soaking time for 1 and 2 h heating times in alkali treatments of *G. cliftonii* strongly influenced the agar characteristics and resulted in considerable agar loss. Although the yields obtained for these treatments were higher than those required by the industry (>8 %db) (Armisen, 1995), they were lower compared to those reported for *G. cliftonii* native agar yield (52 %db) (Byrne *et al.*, 2002) and to the yields obtained from trials in Chapters 3 to 8 (with or without

alkali treatment). The agar properties were similar to those reported for native agar from various *Gracilaria* species as discussed in Chapters 3 to 8 (Arvizu-Higuera *et al.*, 2008; Freile-Pelegrin & Murano, 2005; Freile-Pelegrin & Robledo, 1997a; Miller *et al.*, 1994; Nishinari & Watase, 1983).

Agar yield without alkali treatment at different soaking times and heating times was similar to that obtained for *G. edulis* (21.8 %db) (Durairatnam, 1987) and *G. verrucosa* (21.1 %db) (Hurtado-Ponce & Umezaki, 1988). The agar yields were similar to alkali treated *G. cornea* (Freile-Pelegrin & Robledo, 1997b), *G. cervicornis*, *G. blodgettii* and *G. crassissima* (13-26 %db) at alkali concentrations of 0.5 and 1.0 % (Freile-Pelegrin & Murano, 2005). The difference in yields is related to the difference in temperature, time of soaking and extraction process as previously described (Armisen & Galatas, 1987; Arvizu-Higuera *et al.*, 2008). The lack of significant differences in agar yield with both heating times for 1-3 h soaked samples indicates that alkali concentration is an important factor affecting the yield. The agar yield decreased significantly due to alkali treatment which could be due to the degradation of polysaccharides by alkali during the process, as demonstrated for *G. crassissima* (Freile-Pelegrin & Murano, 2005). Agar yield decreased with increasing soaking time indicates that it is a yield limiting factor. The decrease in agar yield due to soaking could be due to the breaking of hydrogen bonding in the polysaccharide structure, thus resulting in diffusion of some agar into the water.

Agar loss from *G. cliftonii* samples due to the alkali treatment with both heating times was greater than 30 %db while without alkali treatment and 1 h heating it was 29.5 %db. The combination of both alkali and soaking resulted in considerable agar loss due to the loss of alkali-labile sulphates which might have been lost during the process (Buriyo & Kivaisi, 2003) which suggests that *G. cliftonii* contains a large quantities of these sulphates. Various researchers have reported the reduction in agar yield but have not reported the agar loss or the factors initiating or responsible for this loss (Arvizu-Higuera *et al.*, 2008; Freile-Pelegrin & Murano, 2005; Freile-Pelegrin & Robledo, 1997b; Nishinari & Watase, 1983) but in general the loss due to alkali treatment is well established. Buriyo and Kivaisi (2003) reported an agar loss of up to 50% from *G. salicornia* grown in field conditions. Armisen (1987) also suggested that alkali treatment of *Gracilaria* should be carried out in standardised conditions as this process can result in agar loss due to the extraction process.

The gel strength obtained with different alkali treatments was similar to the gel strength for native agar from various other *Gracilaria* species (Falshaw *et al.*, 1999; Freile-Pelegrin & Murano, 2005; Orduña-Rojas *et al.*, 2008; Santos & Doty, 1983). In the present study, there was no significant improvement in the gel strength with alkali treatment unlike for *G. cornea* (Freile-Pelegrin & Robledo, 1997b), *G. cervicornis* and *G. blodgettii* (Freile-Pelegrin & Murano, 2005), nevertheless, the extraction process and species were different in both the studies. However, Freile-Pelegrin (2005) reported decrease in gel strength for *G. crassissima* due to diffusion of agar and alteration of structure which could be a possible reason for the low gel strength in the present study also. In addition, the high gel strength for samples with or without alkali treatment with 2 h soaking followed by 1 h heating time or 1 h soaking followed by 2 h heating time indicates that treatment variables like heating time and soaking time do influence the gel strength and have to be identified and then optimised for *G. cliftonii*. The alkali treatment variables used in the present study also indicate that another factor affecting the gel strength could be that alkali treatment of *G. cliftonii* requires a total time of 3 h for achieving significant gel strength. It has been suggested by Armisen (1987) that alkali treatment and extraction is species specific and is dependent on different treatment parameters. The high sulphate content of agar from different alkali concentration, soaking and heating time combinations might also be a major factor affecting the agar properties. Various researchers have reported the sulphate content of agar bears a negative correlation to gel strength (Andriamanananatony *et al.*, 2007; Craigie & Jurgens, 1989; Freile-Pelegrin & Murano, 2005; Freile-Pelegrin & Robledo, 1997b; Wilkinson *et al.*, 2000). In the present study, high sulphate content of agar from *G. cliftonii* species and the lack of significant difference for certain combinations of alkali treatment suggest it could be responsible for the low gel strength of agar. However, results have to be confirmed as agar structure from this species as well as 3,6-anhydro-L-galactose content is not known.

The decrease in gelling temperature and melting point of agar with different alkali concentrations at both heating times with longer soaking time could be related to molecular weight and molecular weight distribution in agar as these parameters strongly affect the gelling properties (Freile-Pelegrin & Murano, 2005). Agar contains methoxyl, sulphate and phosphate groups which during alkali treatment might show cross bonding or loss, resulting in shift or decrease in molecular weight.

The gelling temperature is reported to be positively correlated with the methoxyl content of agar (Andriamanananattonio *et al.*, 2007; Guiseley, 1970; Rebello *et al.*, 1997) while melting point is related to pyruvic acid content (Andriamanananattonio *et al.*, 2007; Lahaye & Rochas, 1991; Murano, 1995; Young *et al.*, 1971), both of which change after alkali treatment. In addition, soaking and heating the samples might have changed the methoxyl and pyruvic acid content and location as these groups are heat sensitive. In addition, the longer soaking time would have resulted in agar diffusion and loss of hydrogen bonding thus reducing gelling temperature and melting point.

In summary, agar yield and properties are reported to be influenced by structure of the agar (Andriamanananattonio *et al.*, 2007; Craigie & Jurgens, 1989; Duckworth & Yaphe, 1971; Lahaye & Rochas, 1991; Lai & Lii, 1997; Murano, 1995), methoxyl content (affects gelling temperature) (Guiseley, 1970; Rebello *et al.*, 1997), pyruvic acid content (affects melting point) (Andriamanananattonio *et al.*, 2007; Geresh & Arad, 1991; Lahaye & Rochas, 1991; Young *et al.*, 1971) and sulphate position and content (affects gel strength) (Andriamanananattonio *et al.*, 2007; Craigie & Jurgens, 1989). However, it is difficult to assign a standard general extraction method valid for all agarophyte to optimise their yield and at the same time obtain a good quality agar for industrial use (Freile-Pelegrin & Robledo, 1997b).

**CHAPTER 10: MODIFICATION OF ALKALI TREATMENT -HIGH
ALKALI CONCENTRATION AND HEATING TEMPERATURE**

10.1. INTRODUCTION

Alkali treatment at low concentration with different soaking and heating times resulted in reduction of agar yield and quality, as described in Chapter 8. Alkali treatment variables like concentration, heating and soaking time were tested as they are reported to affect the yield and quality of agar from other *Gracilaria* species (Andriamanananatony *et al.*, 2007; Armisen & Galatas, 1987; Arvizu-Higuera *et al.*, 2008; Li *et al.*, 2008; Orduña-Rojas *et al.*, 2008). Freile-Pelegri and Robledo (1997b) reported that soaking temperature notably influences the agar yield and properties of *G. cornea*.

The present Chapter focuses on the effect of high alkali concentration and heating temperature on the agar characteristics of *G. cliftonii*. It provides a basis to standardise the procedure for *G. cliftonii* agar extraction to obtain a gel with characteristics suitable for the industry.

10.2. METHODOLOGY

10.2.1. Sample and alkali preparation

G. cliftonii collected from natural populations were cleaned, oven dried and used for agar extraction following the methodology described in Chapter 9. Four concentrations, 1, 2, 3 and 5 % alkali were prepared by dissolving analytical grade NaOH (Sigma®) in distilled water.

10.2.2. Alkali treatments

G. cliftonii dried samples (5 g db) in triplicate were soaked in each alkali concentration, 1, 2, 3 and 5 % for 3 h at room temperature in 200 mL conical flasks. The flasks were then placed in a waterbath for 1 h at 60 °C, 70 °C and 80 °C. Native agar was used as the control.

10.2.3. Agar extraction

Alkali removal and agar extraction was performed using the methodology described in Chapter 8. Modifications in the alkali treatment and agar extraction process are shown in Appendix 5.

10.3. RESULTS

10.3.1. Agar yield

At a given temperature different NaOH concentrations had no influence ($p>0.05$) on agar yield of *G. cliftonii* (Figure 10.1). However, different temperatures at NaOH concentrations of 1 to 3 % significantly affected the agar yield. At 70 °C, agar yield from *G. cliftonii* treated with 1 and 3 % NaOH was significantly higher ($p<0.05$) than at 60 °C. At 60 °C, agar yield from *G. cliftonii* treated with 2 % NaOH was significantly lower ($p<0.05$) than 70 and 80 °C. However, temperature had no influence ($p>0.05$) on agar yields when *G. cliftonii* was treated with 5 % NaOH.

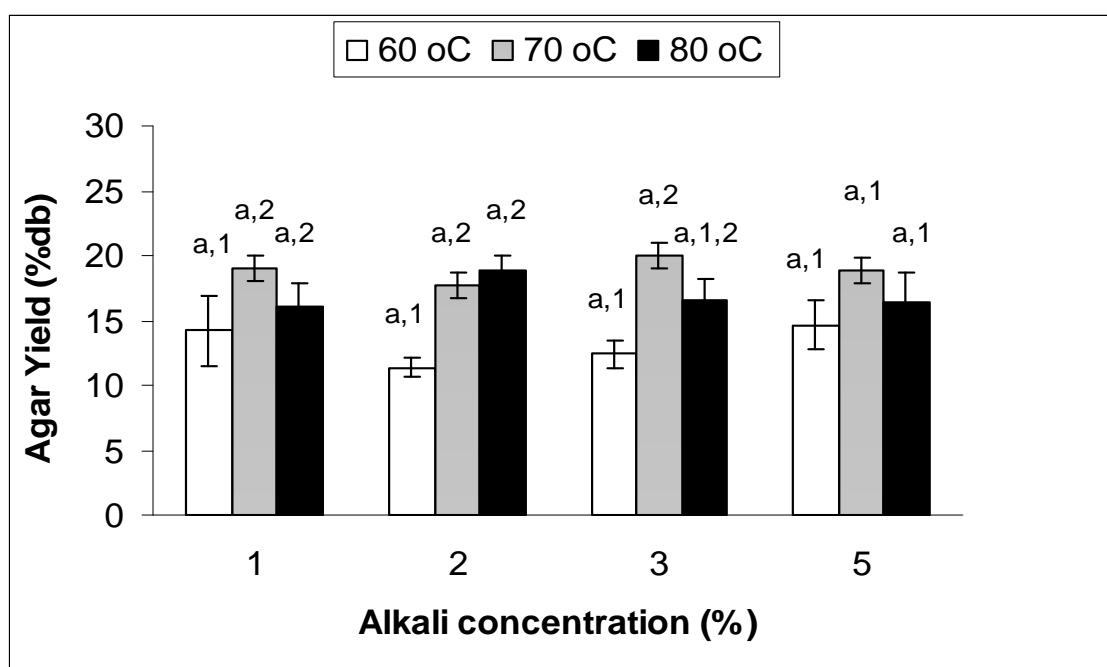


Figure 10.1: Mean agar yield (%db) from *Gracilaria cliftonii* treated at different alkali concentrations and temperature of 60, 70 and 80 °C.

*Error bars represent standard error of mean. Letters a, b, c represent difference between alkali concentrations while numbers 1, 2, 3 represents significant differences between temperatures

10.3.2. Gel strength

The gel strength of agar from alkali treated *G. cliftonii* at different temperatures is shown in Figure 10.2. Gel strength of agar at 60 °C, from 1 to 3 % NaOH treated *G. cliftonii* samples was significantly higher ($p<0.05$) than at 70 °C and 80 °C. In addition, gel strength of agar at 70 °C, from 2 % NaOH treated samples was significantly higher ($p<0.05$) at 80 °C. Gel strength of agar at 70 °C, from 5 % NaOH treated *G. cliftonii* was significantly lower ($p<0.05$) than at 60 and 80 °C. Only at 60 °C, alkali concentration upto 3 % influenced ($p>0.05$) the gel

strength of agar. At 60 °C, gel strength of agar from *G. cliftonii* treated with 3 and 5 % NaOH was significantly higher ($p<0.05$) than 1 and 2 % NaOH concentration. In addition, at 60 °C, gel strength of agar from samples treated with 2 % NaOH was significantly higher ($p<0.05$) than 1 % NaOH. At 80 °C, gel strength of agar from 5 % alkali treated *G. cliftonii* was significantly higher ($p<0.05$) than other alkali concentrations. In addition, gel strength showed a strong positive correlation with alkali concentration ($R^2=0.96$, $p<0.05$).

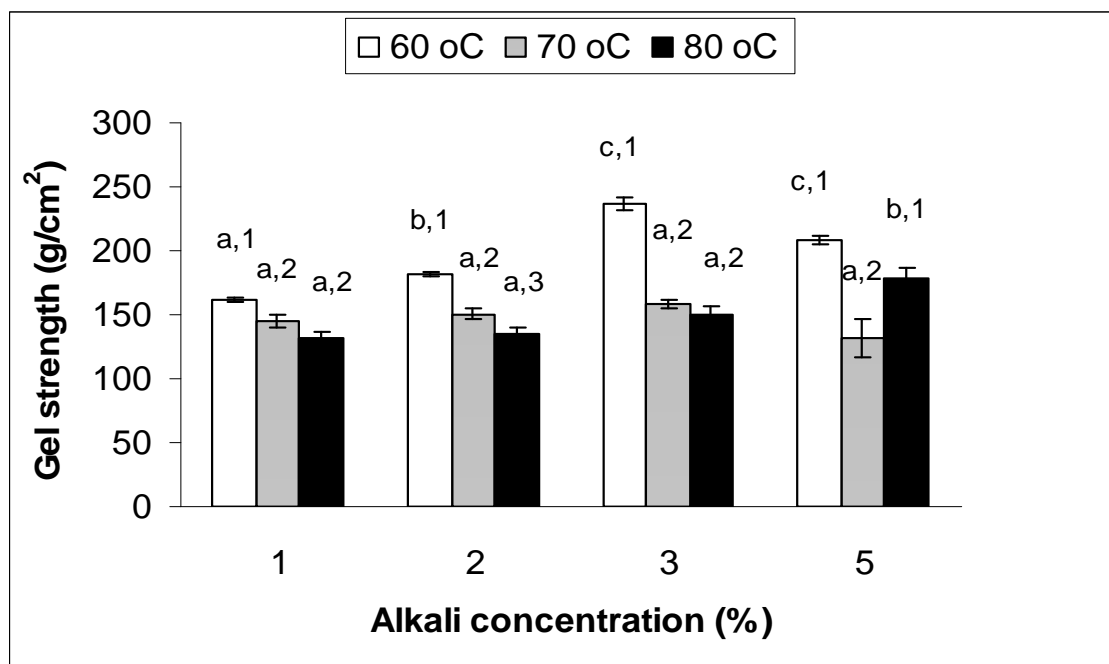


Figure 10.2: Mean gel strength of agar (g.cm^{-2}) from *Gracilaria cliftonii* treated at different alkali concentrations and temperature of 60, 70 and 80 °C.

*Error bars represent standard error of mean. Letters a, b, c represent difference between alkali concentrations while numbers 1, 2, 3 represents significant differences between temperatures

10.3.3. Sulphate content

Sulphate content of agar from alkali treated *G. cliftonii* at various alkali concentrations and temperature ranged between 2.16 to 3.01 % db (Figure 10.3). Alkali concentrations at different temperatures had no influence ($p>0.05$) on the sulphate content of the agar.

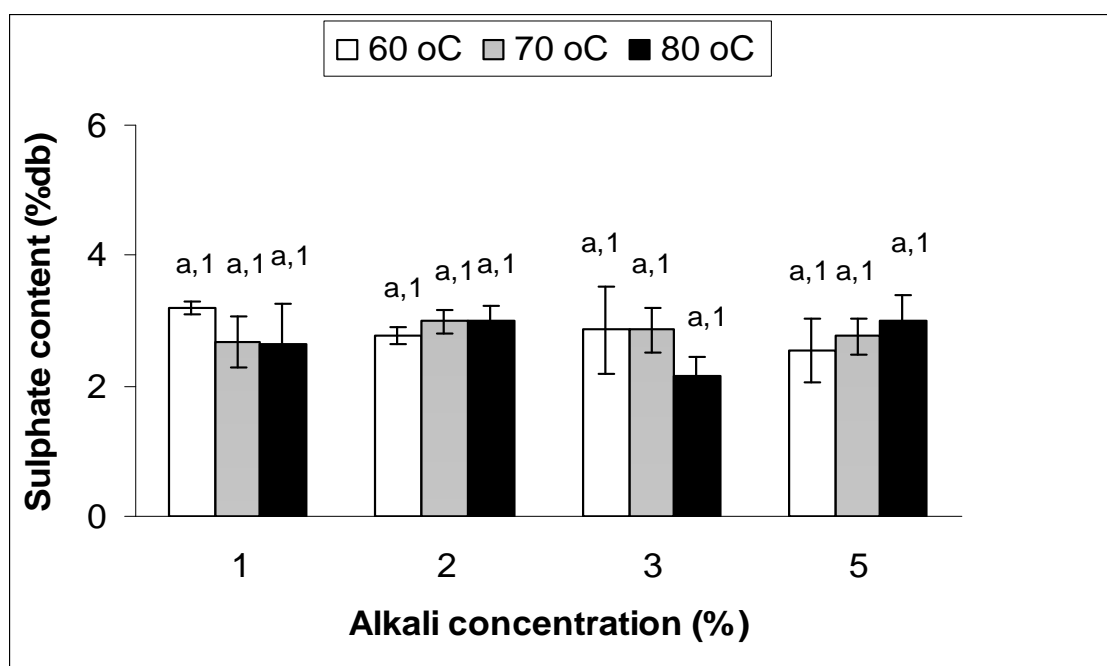


Figure 10.3: Mean sulphate content of agar (%db) from *Gracilaria cliftonii* treated at different alkali concentrations and temperature of 60, 70 and 80 °C.

*Error bars represent standard error of mean. Letters a, b, c represent difference between alkali concentrations while numbers 1, 2, 3 represents significant differences between temperatures

10.3.4. Gelling temperature

The gelling temperatures of agar for alkali treated *G. cliftonii* samples at different soaking temperatures are shown in Figure 10.4. Gelling temperature of agar at 80 °C was significantly higher ($p<0.05$) than at 60 and 70 °C from 1 % NaOH treated *G. cliftonii*. In addition, gelling temperature of agar at 80 °C was significantly higher ($p<0.05$) than at 70 °C from *G. cliftonii* treated with 5 % NaOH. However, gelling temperature of agar at 70 °C was significantly higher ($p<0.05$) than 60 °C and 80 °C from *G. cliftonii* treated with 2 and 3 % NaOH. Except *G. cliftonii* treated with 3 % NaOH, temperature significantly influenced ($p<0.05$) the gelling temperature of agar. At 60 °C, gelling temperature of agar from 1 % NaOH treated *G. cliftonii* was significantly higher ($p<0.05$) than 2 and 5 % NaOH. At 70 °C, gelling temperature of agar from 2 and 3 % NaOH treated samples was significantly higher ($p<0.01$) than 5 % NaOH. At 80 °C, gelling temperature of agar was significantly different ($p<0.05$) at all alkali concentrations.

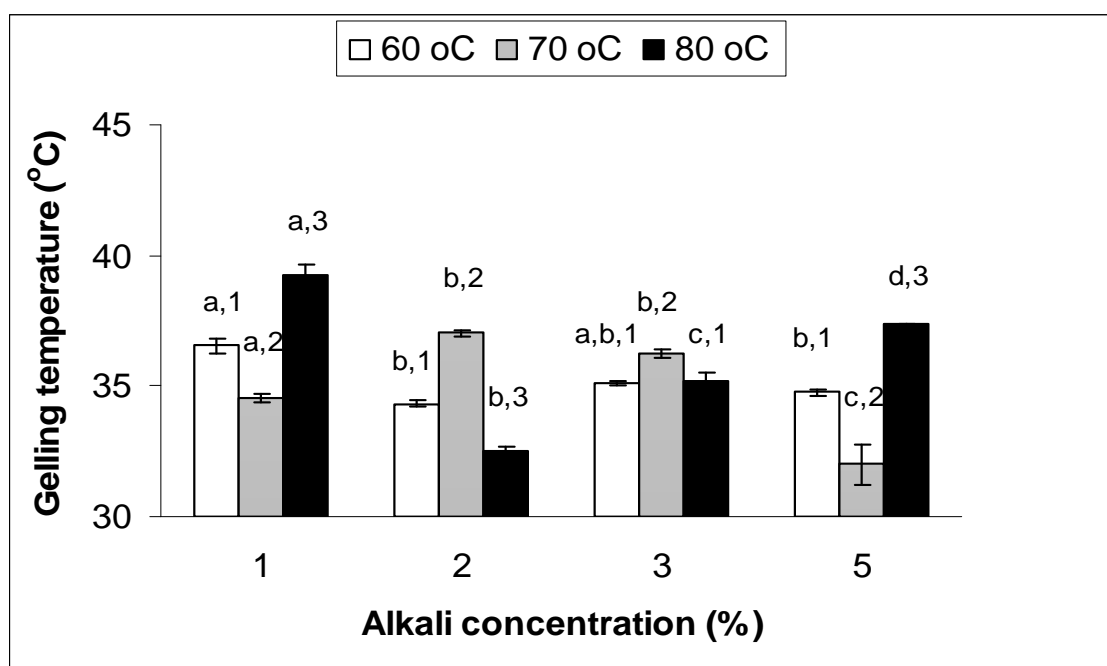


Figure 10.4: Mean gelling temperature (°C) of agar from *Gracilaria cliftonii* treated at different alkali concentrations and temperature of 60, 70 and 80 °C.

*Error bars represent standard error of mean. Letters a, b, c represent difference between alkali concentrations while numbers 1, 2, 3 represents significant differences between temperatures

10.3.5. Melting Point

Melting points of agar for alkali treated samples at different soaking temperatures are shown in Figure 10.5. Melting point of agar was in the range 83.0 °C to 86.0 °C, with minimum observed for *G. cliftonii* treated with 3 % NaOH at 60 °C and maximum for 1 % NaOH at 80 °C (Figure 5). Alkali treatment of *G. cliftonii* at different soaking temperatures did not influenced ($p > 0.05$) the melting point of agar.

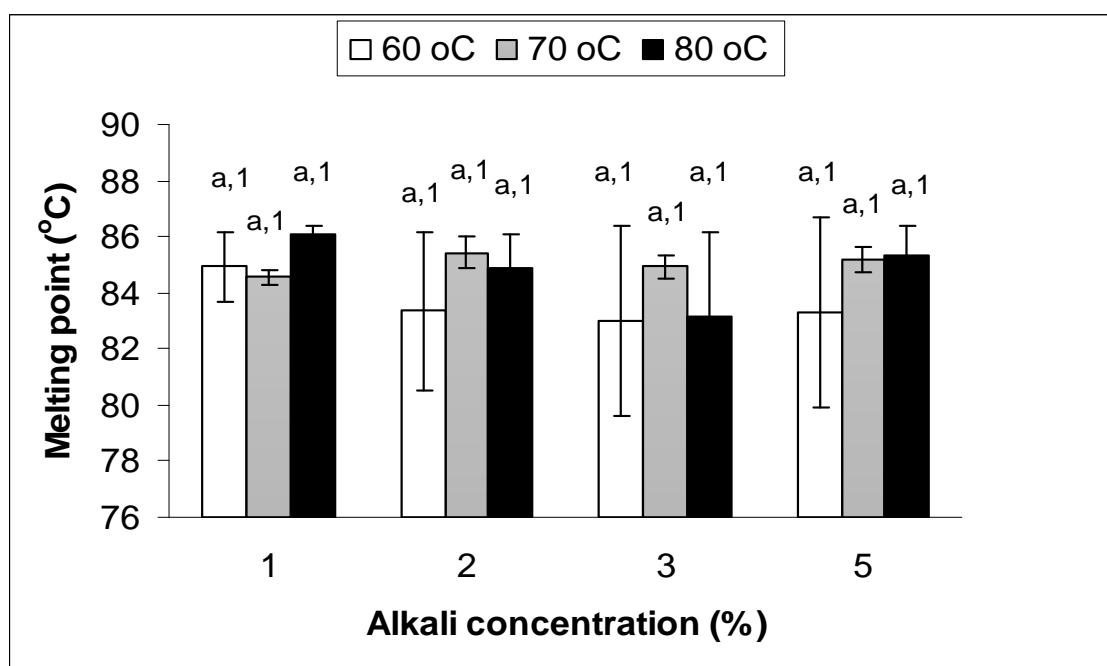


Figure 10.5: Mean melting point (°C) of agar from *Gracilaria cliftonii* treated at different alkali concentrations and temperature of 60, 70 and 80 °C.

*Error bars represent standard error of mean. Letters a, b, c represent difference between alkali concentrations while numbers 1, 2, 3 represents significant differences between temperatures

10.4. DISCUSSION

Alkali treatment of *G. cliftonii* at different soaking temperatures influenced its agar yield and characteristics. However, not all the combinations of alkali concentrations and temperatures had a significant effect on agar characteristics but physical and chemical characteristics of the agar from *G. cliftonii* were improved as compared to native agar. However, the agar characteristics obtained were similar to those reported for native agar from *G. cornea* (Freile-Pelegrin & Robledo, 1997a). Although the agar yields obtained after alkali treatment of *G. cliftonii* were higher than those required by the industry (>8 %db) (Armisen, 1995), but were lower than the reported agar yield of 52 %db (Byrne *et al.*, 2002). The decrease in agar yield of *G. cliftonii* when treated with alkali as compared to native agar can be due to agar diffusion into the water and/or due to the difference in extraction temperatures of agar used in present study. In addition, decrease in agar yield due to alkali treatment compared to native agar suggests that different alkali concentrations in combination with soaking temperatures could have resulted in some degradation of the polysaccharide (Freile-Pelegrin & Murano, 2005; Freile-Pelegrin & Robledo, 1997b).

The agar yield of *G. cliftonii* treated with high alkali concentrations at different temperatures was similar to that observed for *G. edulis* (21.8 %db) (Durairatnam, 1987) and *G. verrucosa* (21.1 %db) (Hurtado-Ponce & Umezaki, 1988). At same NaOH concentrations the agar yields were similar to *G. cornea* at 80 °C (Freile-Pelegrin & Robledo, 1997b). Agar yield obtained at 80 °C was also similar to *G. cervicornis*, *G. blodgettii* and *G. crassissima* (13-26 %db) at 3 and 5 % NaOH concentrations. The difference in agar yield could be related to the differences in used variables like alkali concentration, temperature and time for extraction process (Armisen & Galatas, 1987; Arvizu-Higuera *et al.*, 2008).

The gel strength achieved with different alkali concentrations was similar to the gel strength for native agar from various other *Gracilaria* species (Falshaw *et al.*, 1999; Freile-Pelegrin & Murano, 2005; Orduña-Rojas *et al.*, 2008; Santos & Doty, 1983). Freile-Pelegrin and Robledo (1997b) reported increase in gel strength in *G. cornea* after treatment with 3 and 5 % NaOH concentrations than gel strength of native agar. In the present study, there was no significant improvement in the gel strength with alkali treatment at a given temperature, which can be due to difference in extraction process and/or species. However, the strong positive correlation between the gel strength and alkali concentration is in agreement with the various studies (Freile-Pelegrin & Murano, 2005; Freile-Pelegrin & Robledo, 1997b) and can be due to the elimination of the sulphate ester at C-6 of the L-galactose to increase the 3, 6-anhydro-L-galactose content thereby improving the gelling properties (Murano, 1995).

Alkali treatment converts L-galactose-6-sulphate to 3, 6-anhydro-L-galactose (Duckworth *et al.*, 1971; Freile-Pelegrin & Murano, 2005; Freile-Pelegrin & Robledo, 1997b; Orduña-Rojas *et al.*, 2008), which is responsible for the enhancement of the gel forming ability but in *G. cliftonii* this conversion may not occur. Sulphate content was lower than other *Gracilaria* species but no relation was observed with gel strength with different alkali concentrations at any temperature.

The variation in gelling temperature due to the treatment with the various alkali concentrations and at different soaking temperatures can be related to molecular weight and molecular weight distribution in agar as these parameters strongly affect the gelling properties (Freile-Pelegrin & Murano, 2005). In addition, the methoxyl content and location is reported to affect the gelling temperature of agar (Andriamanananatony *et al.*, 2007; Guiseley, 1970; Rebello *et al.*, 1997) which was

not analysed in present study. However, gelling temperature of agar was similar to *G. cervicornis* (Freile-Pelegrin & Murano, 2005), *G. corticata* (Oyieke, 1993), *G. crassa* (Oyieke, 1993), *G. millardetii* (Oyieke, 1993) and *G. salicornia* (Oyieke, 1993). The melting point was not affected by the alkali concentration at different temperatures but was within the range specified by US Pharmacopoeia (80-85 °C) suggesting possible applications for food and pharmaceutical industry, especially for products that require sterilization. In addition, melting point was similar to *G. tenuistipitata* (Montaño *et al.*, 1999), *G. arcuata* (Montaño *et al.*, 1999), *G. corticata* (Oyieke, 1993), *G. crassa* (Oyieke, 1993), *G. millardetii* (Oyieke, 1993) and *G. salicornia* (Oyieke, 1993). Melting point is reported to be positively correlated to pyruvic acid content of agar (Young *et al.*, 1971), however further investigation is required as this factor was not analysed in present study.

In summary, at a given temperature, there was no effect of alkali treatment on agar properties suggesting different requirements of alkali concentration and temperature for *G. cliftonii*. However, since the agar characteristics can depend on other extraction variables and internal characteristics of the species it is impossible to establish a general extraction method for agarophytes to obtain a high quality agar for industrial use (Freile-Pelegrin & Robledo, 1997b). In our study, the gel strength and sulphate content obtained from agar treated with 3 % alkali at 70 °C are in the ranges required by the Asian food industry (gel strength for striped form agar in range 150-450 g.cm⁻² (1.5 %w/v gel) and sulphate content less than 4 %db, usually 1.5–2.5 %db (Armisen, 1995)). The results obtained in this and previous Chapters suggests *G. cliftonii* treated with different alkali concentrations (high or low), soaking times, treatment time and soaking temperature considerably reduce the agar characteristics. The purpose of alkali treatment prior to agar extraction is to improve the gel characteristics due to the conversion of L-galactose-6-sulphate to 3,6-anhydro-Lgalactose (Duckworth *et al.*, 1971; Freile-Pelegrin & Murano, 2005; Freile-Pelegrin & Robledo, 1997b; Orduña-Rojas *et al.*, 2008), which is responsible for the enhancement of the gel forming ability. However, the gel strength of agar from *G. cliftonii* showed no significant improvement in gel strength and other properties. The agar properties are also dependent on different variables in the extraction process. Therefore, they should be tested without alkali treatment to observe the possible enhancement of properties.

**CHAPTER 11: MODIFICATION OF AGAR EXTRACTION
PROCESS FOR *GRACILARIA CLIFTONII***

11.1. INTRODUCTION

In Chapters 8 to 10, alkali treatment of *G. cliftonii* with different variables like alkali concentration, soaking time, heating time and treatment temperature reduced the agar yield and adversely affected agar properties. The application of agar in different products depends on its chemical composition (sulphate, methoxyl, sugar contents) which can be significantly affected by the variables of the extraction process (Pereira-Pacheco *et al.*, 2007). The general methodology established for agar extraction consists of leaching the dry *Gracilaria* in boiling water, filtering off the extract, and separating the agar by freezing and thawing to eliminate the water (Armisen & Galatas, 1987). Although the general steps in the extraction process are known, the extraction variables and methodologies used differ among authors (Chapter 1). Therefore, it is necessary to standardise the extraction process to optimise the agar yield and quality. The influence of extraction temperature and extraction time on agar characteristics has been reported for *Gracilaria* species (Arvizu-Higuera *et al.*, 2008; Hurtado-Ponce, 1992; Pereira-Pacheco *et al.*, 2007). A notable reduction in agar yield and decrease in gel strength are frequently associated with elevated extraction temperature and prolonged extraction time (Arvizu-Higuera *et al.*, 2008; Hurtado-Ponce, 1992; Pereira-Pacheco *et al.*, 2007).

In the present chapter, *G. cliftonii* agar extraction process was re-evaluated to optimise agar yield by investigating the effects of five extraction variables *viz.* soaking time, soaking temperature, seaweed-water ratio, time and temperature of extraction. The chemical and physical properties (gel strength, gelling temperature, melting point and sulphate content) of agar were measured to determine the effect of each variable tested.

11.2. MATERIAL AND METHODS

11.2.1. Sample preparation

Preparation of samples is described in Chapter 2.

11.2.2. Variables tested and agar extraction

11.2.2.1. Soaking time

The first lot of 'Clean seaweed' was divided into 12 samples of 5 g (dry weight) and 3 samples each from these 12 samples were soaked for 1, 2 and 3 h at room temperature (25 °C) in triplicate to hydrate the seaweeds. The remaining 3 samples without soaking (0 h) were used as the control and also in triplicate. Extraction was carried out by boiling all samples for 2.5 h in 250 mL conical flasks with distilled water at 90 °C in a waterbath.

11.2.2.2. Soaking temperature

The second lot of 'Clean seaweed' was divided into 12 samples of 5 g (dry weight) and 3 samples each from these 12 samples were soaked for 2 h at three different temperatures (30 °C, 35 °C and 40 °C) in triplicate. Three remaining samples were soaked at room temperature (25 °C) and used as the control in triplicate. Extraction was carried out by boiling all samples for 2.5 h in 250 mL conical flasks with distilled water at 90 °C in a waterbath.

11.2.2.3. Seaweed to water ratio

The third lot of 'Clean seaweed' was divided into 12 samples of 5 g (dry weight) and soaked for 2 h at 30 °C in different volume of water to represent variable seaweed-water ratios. These four different seaweed-water ratios were 1:50, 1:100, 1:150 and 1:200. All of them were used in triplicate and then transferred to a waterbath for agar extraction. Extraction was carried out by boiling all the samples for 2.5 h in 250 mL conical flasks with distilled water at 90 °C in a water bath.

11.2.2.4. Extraction temperature

The fourth lot of 'Clean seaweed' was divided into 12 samples of 5 g (dry weight) each and soaked for 2 h at 30 °C with a seaweed-water ratio of 1:150. Extraction was carried out at four different temperatures of 70 °C, 80 °C, 90 °C and 100 °C in triplicate by boiling the samples for 2.5 h in waterbaths.

11.2.2.5. Extraction time

The fifth and last lot of 'Clean seaweed' was divided into 12 samples of 5 g (dry weight) and soaked for 2 h at 30 °C with a seaweed-water ratio of 1:150. Extraction from the samples was carried out at 100 °C at five different times of 1.0, 1.5, 2.0, 2.5, 3.0 h in triplicate in a waterbath.

11.3. RESULTS

11.3.1. Soaking time and temperature

Soaking time and temperature significantly influenced agar yield and gelling temperature (Table 11.1 and Table 11.2). The maximum agar yield was obtained with soaking time of 1 and 2 h and was significantly higher ($p < 0.05$) than 3 h soaking time. Gelling temperature of agar with 3 h soaking time was significantly higher ($p < 0.05$) than other soaking times. Agar yield showed strong negative correlation ($R^2 = -0.8$, $p < 0.05$) with sulphate content with increasing soaking time. Agar yield at soaking temperature of 30 °C was significantly higher ($p < 0.05$) than other soaking temperatures. Gelling temperature of agar at 30 °C was significantly higher ($p < 0.05$) than 40 °C. The sulphate content of agar at 35 °C was significantly higher ($p < 0.05$) than control. Gel strength showed negative correlation ($R^2 = -0.7$, respectively, $p < 0.05$) with sulphate content with increasing soaking temperature.

Table 11.1: Agar yield and properties (Mean \pm S.E.) from *Gracilaria cliftonii* at soaking time 0, 1, 2 and 3 h.

	Soaking time (h)			
	0	1	2	3
Agar yield (%db)	59.8 \pm 1.0 ^{a,b}	61.2 \pm 0.1 ^a	61.2 \pm 1.0 ^a	54.9 \pm 2.6 ^b
Gel strength (g/cm²)	171.8 \pm 8 ^a	159.4 \pm 14 ^a	170.9 \pm 8 ^a	180.8 \pm 12 ^a
Melting point (°C)	86.5 \pm 0.9 ^a	87.1 \pm 1.5 ^a	86.7 \pm 0.7 ^a	85.8 \pm 0.6 ^a
Gelling temperature (°C)	33.1 \pm 0.2 ^a	33.1 \pm 0.3 ^a	33.2 \pm 0.3 ^a	37.9 \pm 0.5 ^b
Sulphate (%db)	7.1 \pm 0.3 ^a	6.9 \pm 0.5 ^a	6.8 \pm 0.0 ^a	7.1 \pm 0.9 ^a

*Different letters (a, b, c) are significantly different at a level of $p < 0.05$

Table 11.2 : Agar yield and properties (Mean \pm S.E.) from *Gracilaria cliftonii* at soaking temperature of 25, 30, 35 and 40 °C

	Soaking temperature (°C)			
	25	30	35	40
Agar yield (%db)	52.7 \pm 0.2 ^a	62.3 \pm 0.7 ^b	49.7 \pm 1.9 ^a	51.3 \pm 1.2 ^a
Gel strength (g/cm²)	126.3 \pm 6 ^a	119.0 \pm 4 ^a	117.7 \pm 1 ^a	123.3 \pm 5 ^a
Melting point (°C)	81.8 \pm 3.5 ^a	86.7 \pm 0.3 ^a	88.0 \pm 2.0 ^a	86.5 \pm 1.7 ^a
Gelling temperature (°C)	35.0 \pm 1.2 ^{a,b}	35.5 \pm 1.0 ^a	34.9 \pm 1.3 ^{a,b}	32.0 \pm 0.4 ^b
Sulphate (%db)	5.1 \pm 1.1 ^a	7.5 \pm 0.7 ^{a,b}	8.2 \pm 0.9 ^b	7.7 \pm 0.5 ^{a,b}

*Different letters (a, b, c) are significantly different at a level of $p < 0.05$

11.3.2. Seaweed to water ratio

The variation in the agar yield and properties with various weed-water ratios is shown in Table 11.3. The agar yield with seaweed-water ratio of 1:150 was significantly higher ($p < 0.05$) than other ratios. Agar yield with weed-water ratio of 1:100 was significantly lower ($p < 0.05$) than other ratios. Gel strength of agar with seaweed-water ratio of 1:100 was significantly lower ($p < 0.05$) than seaweed-water ratio of 1:200. With increasing seaweed-water ratio Gel strength was negatively correlated ($R^2 = -0.8$, $p < 0.05$) with melting point while gelling temperature was negatively correlated ($R^2 = -0.9$, $p < 0.05$) with sulphate content.

Table 11.3: Agar yield and properties (Mean \pm S.E.) from *Gracilaria cliftonii* with seaweed-water ratio of 1:50, 1:100, 1:150 and 1:200.

	Seaweed to water ratio			
	1:50	1:100	1:150	1:200
Agar yield (%db)	52.6 \pm 0.4 ^a	50.5 \pm 0.7 ^b	57.8 \pm 0.2 ^c	53.7 \pm 0.2 ^a
Gel strength (g/cm²)	133.0 \pm 9 ^{a,b}	124.3 \pm 5 ^a	143.1 \pm 11 ^{a,b}	159.9 \pm 14 ^b
Melting point (°C)	85.7 \pm 1.2 ^a	87.1 \pm 0.8 ^a	85.6 \pm 0.4 ^a	84.9 \pm 1.8 ^a
Gelling temperature (°C)	34.7 \pm 0.7 ^a	33.2 \pm 0.2 ^a	33.1 \pm 0.4 ^a	34.8 \pm 1.5 ^a
Sulphate (%db)	5.9 \pm 1.5 ^a	7.2 \pm 0.7 ^a	7.1 \pm 1.3 ^a	4.8 \pm 0.9 ^a

*Different letters (a, b, c) are significantly different at a level of $p < 0.05$

11.3.3. Extraction temperature and time

The effect of extraction temperature and time on agar yield and properties is shown in Table 11.4 and 11.5. The agar yield was significantly different ($p < 0.05$) with each other at different extraction temperatures. Maximum agar yield was obtained at extraction temperature of 100 °C and minimum at 70 °C. Agar yield and melting point showed strong positive correlation ($R^2 = 0.98$ and $R^2 = 0.87$ respectively, $p < 0.05$) with extraction temperatures. With increasing extraction temperature, agar yield was positive correlated ($R^2 = 0.7$, $p < 0.05$) while gel strength was negatively correlated ($r^2 = -0.7$, $p < 0.05$) to sulphate content.

The agar yield with 1 and 1.5 h extraction time was significantly lower ($p < 0.001$) than the other extraction time. The agar yield with extraction times of 2, 2.5 and 3 h was not significantly different with each other. Gel strength of agar with 1.5 and 3.0 h extraction time was significantly higher ($p < 0.05$) than with 1 h extraction time. Melting point of agar with 1 h extraction time was significantly lower ($p < 0.05$) than extraction time of 1.5, 2.0 and 2.5 h. Gelling temperature with 1 h extraction time was significantly lower than 1.5 and 2.5 h ($p < 0.05$) extraction time. Agar yield showed strong positive correlation ($R^2 = 0.8$, $p < 0.05$) with extraction time. With increasing extraction time, gelling temperature showed strong positive correlation ($R^2 = 0.9$, $p < 0.05$) with melting point.

Table 11.4: Agar yield and properties (Mean \pm S.E.) from *Gracilaria cliftonii* at extraction temperature of 70, 80, 90 and 100 °C

	Extraction temperature (°C)			
	70	80	90	100
Agar yield (%db)	48.0 \pm 0.6 ^a	51.9 \pm 0.6 ^b	54.8 \pm 0.2 ^c	60.6 \pm 0.4 ^d
Gel strength (g/cm²)	202.1 \pm 17 ^a	246.7 \pm 28 ^a	233.5 \pm 18 ^a	186.8 \pm 8 ^a
Melting point (°C)	85.9 \pm 0.7 ^a	86.8 \pm 0.1 ^a	87.0 \pm 0.8 ^a	87.2 \pm 0.7 ^a
Gelling temperature (°C)	37.6 \pm 1.8 ^a	39.2 \pm 1.3 ^a	38.8 \pm 1.4 ^a	38.3 \pm 0.8 ^a
Sulphate (%db)	5.7 \pm 0.9 ^a	5.0 \pm 0.8 ^a	5.8 \pm 1.4 ^a	7.5 \pm 0.6 ^a

*Different letters (a, b, c) are significantly different at a level of $p < 0.05$

Table 11.5: Agar yield and properties (Mean \pm S.E.) from *Gracilaria cliftonii* at extraction time 1, 1.5, 2, 2.5 and 3 h

	Extraction time (h)				
	1	1.5	2	2.5	3
Agar yield (%db)	38.2 \pm 1.3 ^a	39.1 \pm 1.5 ^a	54.8 \pm 0.2 ^b	55.6 \pm 0.2 ^b	56.7 \pm 0.4 ^b
Gel strength (g/cm²)	109 \pm 2 ^a	146 \pm 7 ^b	138 \pm 18 ^{a,b}	126 \pm 10 ^{a,b}	147 \pm 17 ^b
Melting point (°C)	76.3 \pm 5 ^a	85.9 \pm 2 ^b	84.5 \pm 1 ^b	84.4 \pm 1 ^b	82.0 \pm 1 ^{a,b}
Gelling temp. (°C)	30.8 \pm 1 ^a	34.0 \pm 1 ^b	33.1 \pm 1 ^{a,b}	34.4 \pm 1 ^b	33.1 \pm 1 ^{a,b}
Sulphate (%db)	7.8 \pm 0.1 ^a	6.4 \pm 0.6 ^a	7.9 \pm 0.6 ^a	6.9 \pm 1.4 ^a	6.7 \pm 0.8 ^a

*Different letters (a, b, c) are significantly different at a level of $p < 0.05$

11.4. DISCUSSION

As the agar extraction method has to be standardised for each species considering maximise their yield and at the same time obtain a good quality agar for industrial use (Freile-Pelegrin & Robledo, 1997b). The present study is the first attempt to test different variables in agar extraction process of *G. cliftonii* to optimise yield and to investigate impact of these variables on agar characteristics.

11.4.1. Soaking time and temperature

The purpose of soaking is to hydrate the seaweeds and ease the availability of soluble polysaccharides. Effects of soaking duration and soaking temperature have not been reported for any *Gracilaria* species but different researchers over years have used different soaking times for seaweeds prior to extraction (Arvizu-Higuera *et al.*, 2008; Orduña-Rojas *et al.*, 2008). The main constituents of seaweeds are polysaccharides, which are hydrophilic polymers. Water reacts with external and internal polysaccharides of seaweeds by hydrogen bonding and water-holding capacity of seaweeds also varies according to the species (Jiménez-Escrig & Sánchez-Muniz, 2000). The longer soaking time (3 h) can result in diffusion of some agar into water resulting in lower yields. In addition, negative correlation between agar yield and sulphate content with increase in soaking time indicates alteration in agar structure as sulphate forms important part of agar molecular chain. The role of sulphate on agar yield due to change in agar structure has been reported in various studies (Armisen & Galatas, 2000; Falshaw *et al.*, 1999; Marinho-Soriano *et al.*, 1999; Orduña-Rojas *et al.*, 2008). Longer soaking time also influenced gelling temperature of agar, which was highest at 3 h of soaking in our studies. The high gelling temperature is also related to the change in agar structure due to longer soaking time. The gelling temperature is reported to be positively correlated to the methoxyl content of agar (Guiseley, 1970; Rebello *et al.*, 1997) which in present study was not determined.

The soaking temperature can also influence the agar yield and properties as the average ambient temperature during the culture of *Gracilaria* varies from various geographic region and time of the year. Different soaking time strongly influenced the agar yield, gelling temperature and sulphate content of agar. At higher soaking temperatures (35 and 40 °C) lower yield could be due to some diffusion of agar in water. The high yield at 30 °C is related to changes in agar structure thus allowing the release of most polysaccharides when extracted at this temperature. The polysaccharides are hydrophilic in nature and have intracellular hydrogen bonding which can be broken due to mild heat thus altering the structure. This can also explain the high gelling temperature of agar with soaking temperature of 30 °C as compared to 40 °C. The gelling temperature of agar is reported to increase with increase in methoxyl content (Andriamanananatony *et al.*, 2007; Duckworth & Yaphe, 1971; Guiseley, 1970). Higher temperature (40 °C) can rupture the agar-methoxyl

bonding resulting in lower gelling temperature. In addition, higher sulphate content with higher soaking temperature can affect the gelling temperature as reported in various studies (Andriamanananatony *et al.*, 2007; Arvizu-Higuera *et al.*, 2008; Craigie & Jurgens, 1989; Navarro *et al.*, 2007; Orduña-Rojas *et al.*, 2008). The increase in sulphate content with soaking temperature can be related to heat induced changes in the molecular structure of agar.

11.4.2. Seaweed to water ratio

Effects of seaweed-water ratio during agar extraction process on agar properties has not been published for any *Gracilaria* species but different researchers over years have used different ratios for agar extraction (Araño *et al.*, 2000; Arvizu-Higuera *et al.*, 2008; Freile-Pelegrin & Murano, 2005; Orduña-Rojas *et al.*, 2008; Tako *et al.*, 1999). Agar yield and gel strength were significantly different with different volumes of water for same amount of *G. cliftonii*. The greater the volume of water better is the swelling of seaweed thus, allowing the agar to be extracted easily. Dried marine algae can swell to about 20 times of their dry matter volume when exposed to water (Jiménez-Escrig & Sánchez-Muniz, 2000). Agar yield with seaweed-water ratio of 1:200 was lower which may be due to the excess of water resulting in diffusion of agar but resulted in higher gel strength. The higher gel strength with seaweed-water ratio of 1:200 could be attributed to the changes in agar structure and can be explained with low sulphate content (Andriamanananatony *et al.*, 2007; Arvizu-Higuera *et al.*, 2008; Craigie & Jurgens, 1989; Duckworth & Yaphe, 1971; Nunn *et al.*, 1973; Orduña-Rojas *et al.*, 2008). Gelling temperature showed a negative correlation with sulphate and is reported to be affected by the methoxyl content in agar (Andriamanananatony *et al.*, 2007; Arvizu-Higuera *et al.*, 2008; Craigie & Jurgens, 1989; Navarro *et al.*, 2007; Orduña-Rojas *et al.*, 2008). The agar structure change due to increasing water volume can result due to change in location of methoxyl group and sulphate in agar structure (Craigie & Jurgens, 1989; Lloyd *et al.*, 1961). In addition, melting points negative correlation to gel strength also indicates change in structure and may be due change in pyruvic acid content of agar. Melting point is reported to be positively correlated to the pyruvic acid content and alters the structure of agar (Young *et al.*, 1971).

11.4.3. Extraction temperature

Different extraction temperatures significantly influenced the agar yield of *G. cliftonii* but also indicated a significant amount of agar is extracted even at low

temperature (70 °C). The agar extraction temperature from *Gracilaria* species is reported to be 85-100 °C (Arvizu-Higuera *et al.*, 2008; Marinho-Soriano & Bourret, 2005; Meena *et al.*, 2006; Orduña-Rojas *et al.*, 2008; Santelices & Doty, 1989) but there is no literature reporting influence of extraction temperature on agar yield and properties. Various authors have reported alkali treatment temperatures of 80 to 100 °C for *Gracilaria* prior to extraction and observed significant decrease in agar yield due to the process (Armisen & Galatas, 2000; Falshaw *et al.*, 1999; Freile-Pelegrin & Murano, 2005; Freile-Pelegrin & Robledo, 1997b; Marinho-Soriano, 2001). In the present study, agar yield observed at different extraction temperatures can be correlated to the decrease in agar yield during alkali treatment of *Gracilaria*. Agar yield of 48 %db at extraction temperature of 70 °C indicate that considerable amount of agar is being extracted at low temperatures therefore while treating *Gracilaria* with alkali at even 70 °C, agar gets extracted and can be lost when discarding excess alkali after the treatment. The agar yield was different for various extraction temperatures with maximum at 100 °C which is in agreement with previous studies (Freile-Pelegrin & Murano, 2005; Freile-Pelegrin & Robledo, 1997b).

Extraction temperature's strong positive correlation with agar yields indicates its strong influence on agar extraction. In addition, melting point also showed positive correlation with extraction temperature indicating the alteration of structure due to higher temperatures in extraction process though the increase is not significant. Gel strength's negative correlation with sulphate and positive correlation with gelling temperature confirms the change in structure due to conversion of L-galactose-6-sulphate to 3,6-anhydro-L-galactose (Duckworth *et al.*, 1971; Freile-Pelegrin & Murano, 2005; Freile-Pelegrin & Robledo, 1997b; Orduña-Rojas *et al.*, 2008).

11.4.4. Extraction time

In present study, extraction time was found to be as one of the important extraction process variables as agar yield and all the properties (except sulphate content) showed significant differences at different extraction times. Agar yield was significantly lower with extraction time of 1 and 1.5 h, which is due to incomplete agar extraction. All the properties were affected with 1 h extraction time indicating that minimum time is required to complete the agar extraction to have desirable properties. The extraction time for maximum yield was 2 to 3 h, which is in confirmation with various agar-based studies. For example, Thomas and

Krishnamurthy (1976) studied the effect of extraction time (1-6 h) on *G. edulis* and found maximum yield with 2- 4 h extraction time, Arvizu-Higuera, et al. (2008), also reports the maximum agar yield with 2.5 h extraction time in *G. vermiculophylla*.

The present result confirms that agar yield is influenced by all the variables used and tested during agar extraction process. It also shows that all the agar properties can be manipulated in terms of soaking time and temperature, weed-water ratio, extraction time and temperature in the extraction process. In addition, similar gel strength can be obtained by either manipulating the extraction process variables or alkali treatment. It is recommended that dry *G. cliftonii* is soaked for 1 h at 30 °C with weed-water ratio of 1:150 and extraction of agar in boiling water at 100 °C for 3 h to obtain maximum yield and high quality.

CHAPTER 12: GENERAL DISCUSSION AND CONCLUSION

12.1. INTRODUCTION

The present study aimed to investigate *G. cliftonii* as a potential species for ISW aquaculture. There is no literature available on the growth rates, effect(s) of biotic and abiotic variables on *G. cliftonii* under culture conditions of OW or ISW. In addition, the chemical composition, physicochemical and agar properties of *G. cliftonii* are also unknown. The present study has demonstrated that *G. cliftonii* could be a possible candidate species for ISW aquaculture. However, its productivity, agar yield and properties need to be carefully considered before a decision to commercially cultivate *G. cliftonii* under ISW conditions is undertaken. The present study has evaluated these parameters and thus will assist in making the decision. In general, the high protein and fibre content of *G. cliftonii* indicates its potential as a health food through direct consumption or as a value added product for the health food industry while the high WRC and ORC of this species make it a potential food additive for bulking foods.

The study has shown that appropriate nutrient media and culture conditions can provide promising growth rates and net yield of *G. cliftonii* in ISW and OW. The agar yield from the culture trials was higher than other congeneric species. Culturing *G. cliftonii* with different commercially available nutrient media and variable N-P-K supplementation resulted in high growth rates and net yield in OW and ISW. This indicates that suitable nutrient composition of the nutrient media is essential for the successful culture of *G. cliftonii*. In addition, growth of *G. cliftonii* was a function of different ionic profiles of ISW and further improvement in growth was achieved when ISW was fortified with K⁺.

Reproductive and vegetative stages of *G. cliftonii* showed different agar yield and properties with or without alkali treatment. Reproductive stages had higher agar content but lower quality as compared to vegetative stage. Alkali treatment of *G. cliftonii* resulted in a decline in the yield and quality of agar. Varying the alkali treatment parameters viz. soaking and heating time also resulted in a loss of yield and quality of agar from different life stages of *G. cliftonii*. Alkali treatment of *G. cliftonii* was modified with variables such as alkali concentration, soaking and heating time and treatment temperature in order to optimise the agar yield and properties. It was demonstrated that varying the alkali treatment parameters resulted

in agar loss but improved the quality of agar. However, the improvement in agar properties was not significant at lower alkali concentrations and the agar loss was significantly higher. Alkali treatment with higher alkali concentrations of 1-5 % alkali at different treatment temperatures resulted in improved agar properties, particularly gel strength, but also resulted in significant agar loss. Agar loss from alkali treatment without significant improvement in agar properties presented the grounds for modification of the overall agar extraction process. Soaking time and temperature, seaweed to water ratio, extraction time and temperature were identified as the major process variables affecting the yield and properties and were modified to optimise the extraction process for optimising yield. Each of the process variables tested resulted in variation in agar yield and properties. It was demonstrated that agar yield and properties can be improved without alkali treatment by varying the extraction process variables alone. The major outcomes of the present research are summarised in Table 12.1.

Table 12.1: Positive (+), negative (-) or neutral (NE) effects of different variables tested on the desired parameters as compared to the natural population or control used within the trial.

Desired parameters	Variables tested						
	Nutrients		Ionic profiles		Stages	Alkali treatment	
	Media	NPK	Raw	Fortified		Soaking, heating	Concentrations, temperature
Growth	+	+	+	+	N/A	N/A	N/A
WRC	+	+	N/A	+	N/A	N/A	N/A
ORC	+	+	N/A	+	N/A	N/A	N/A
Agar yield	+	+	+	+	-	-	-
Gel strength	-	-	-	-	-	-	+
Melting point	NE	NE	NE	NE	-	-	+
Gelling temperature	NE	NE	NE	NE	-	-	+
Sulphate content	NE	NE	NE	NE	NE	NE	+

N/A represents not applicable

12.2. GROWTH

12.2.1. Effect of Nutrients

The use of different commercial nutrient media or nutrient supplementation for culturing *G. cliftonii* had a beneficial effect on the growth parameters in OW and ISW (Chapters 4 and 5). Similar SGR of *G. cliftonii* was achieved in OW and ISW with the same nutrient supply. The use of commercial nutrient media resulted in significantly higher ($p < 0.01$) SGR and net yield of *G. cliftonii* than with N-P-K supplementation in ISW and OW (Table 12.2). Net yield of *G. cliftonii* with nutrient media in ISW was higher than in OW.

Table 12.2: Specific growth rate (SGR) and Net yield of *Gracilaria cliftonii* (Mean \pm S.E.) in ocean water (OW) and inland saline water (ISW) with commercial nutrient media and N-P-K supplementation

NUTRIENT	SGR (%day ⁻¹)		NET YIELD (g.m ⁻² day ⁻¹)	
	OW	ISW	OW	ISW
Commercial Media	0.96 \pm 0.2 ^a	0.92 \pm 0.2 ^a	1.51 \pm 0.4 ^a	2.08 \pm 0.4 ^a
N-P-K Supplementation	0.30 \pm 0.02 ^b	0.28 \pm 0.01 ^b	0.89 \pm 0.03 ^b	0.90 \pm 0.04 ^b

(Letters a, b represent the significant differences between nutrients)

The higher SGR and net yield observed due to application of commercial nutrient media in both water types as compared to inorganic nutrient supplementation could be due to the different nitrogen source provided through commercial nutrients. Various authors have reported nitrogen as a growth limiting nutrient for *Gracilaria* species (Bird *et al.*, 1982; Friedlander, 2001; Hanisak, 1990; Jones *et al.*, 1996; Lignell & Pedersén, 1987; Ryther *et al.*, 1981; Smit, 2002). Nitrogen from different sources has different absorption rates in *G. foliifera* (D'Elia & DeBoer, 1978), *G. tikvahiae* (Hanisak, 1990) and *G. tenuistipitata* (Haglund & Pedersén, 1993), with preference for NH₄-N rather than NO₃-N. However, in the present study the preference of nitrogen could be from NO₃-N (f2 and Walne's) as compared to NH₄-N (N-P-K supplementation). Similarly, lower SGR was observed with Aquasol™ media having urea and NH₄-N as the nitrogen source (Chapter 3). The differential

preference for nitrogen source can also be explained by the fact that the nitrogen absorption rate by *Gracilaria* species is also influenced by its nutrient status (1990) that is the conditions under which *G. cliftonii* was collected, transported and acclimatised before the commencement of the experiments.

An optimum N-P ratio of 10:1 has been reported for different *Gracilaria* species (Asare, 1980b; D'Elia & DeBoer, 1978; Navarro-Angulo & Robledo, 1999). Besides N, P and K there are a wide range of vitamins and minerals required by seaweeds that can become limiting in land-based, low exchange seaweed culture systems (Lobban & Harrison, 1994). The lower SGR of *G. cliftonii* with nutrient supplementation could be due to the absence of vitamins and minerals in both water types. In the present study, commercial nutrient media having an N-P ratio of more than 5 shows higher SGR as compared to nutrient supplementation with an N-P ratio of less than 3. There is no published information on the benefits of K^+ as a nutrient for *G. cliftonii* while the role of vitamins and other minerals is well established for microalgae but not on red seaweeds.

12.2.2. Effect of ionic profiles of ISW

Higher SGR and net yield were obtained with different nutrient media in ISW (Chapters 4 and 5). However, the ionic profile of ISW varies with seasons and geographical location. In addition, the salinity of ISW is influenced by rainfall (Prangnell, 2006; Tantulo, 2007). In order to test the impact of varying ionic profile on *G. cliftonii*, different ionic profiles of ISW were achieved by mixing ISW with OW at salinities of 25 and 35 ppt in order to investigate the role of ions (Chapter 6), particularly K^+ (Chapter 7). In Chapter 7, these mixed water types were fortified with different concentrations of K^+ , to bring it closer to the concentration of K^+ present in OW. *Gracilaria* species are reported to have high salinity tolerance under culture conditions and various studies have demonstrated affect of salinity on growth parameters (Choi *et al.*, 2006; Daugherty & Bird, 1988; Ekman *et al.*, 1991; Israel *et al.*, 1999).

The mean SGR and net yield of *G. cliftonii* obtained with raw and fortified profiles of ISW at salinities of 25 and 35 ppt are shown in Table 12.3. SGR and net yield of *G. cliftonii* with K^+ fortified ionic profiles were significantly higher ($p < 0.01$) than with raw ionic profiles at both salinities. However, the two salinities had no impact on the SGR and net yield of *G. cliftonii*. Nevertheless, the present study indicates the effect of K^+ on seaweed growth is not reported but the present study

indicates its influence on the growth of *G. cliftonii*. Shang (1976) suggested an optimum salinity of 25 ppt for growing *Gracilaria* species and Navarro-Angulo and Robledo (1999) also observed detrimental effects on SGR during extended culture periods for *G. cornea* at 35 ppt. However, in the present study salinity change did not influence the growth parameters of *G. cliftonii* in different water types with or without fortification. Other factors affecting the SGR and net yield of *G. cliftonii* in different ionic profiles with or without potassium fortification can be hair formation, type of tissue, age of plant, its nutritional history and interplant variability (Craigie & Wen, 1984; Lobban & Harrison, 1994) which were not studied in the present research. In addition, cutting the thallus to produce a tissue segments could also have influenced the uptake rate of nutrients and flow of ions (Hatcher, 1977).

Table 12.3: Specific growth rate (SGR) and Net yield of *Gracilaria cliftonii* (Mean \pm S.E.) in different salinities with and without potassium fortification of ISW profiles after one month.

SALINITY	SGR (%day ⁻¹)		NET YIELD (g.m ⁻² day ⁻¹)	
	Raw	Fortified	Raw	Fortified
35 ppt	0.29 \pm 0.1 ^a	1.01 \pm 0.1 ^b	0.84 \pm 0.3 ^a	3.19 \pm 0.4 ^b
25 ppt	0.36 \pm 0.1 ^a	0.99 \pm 0.1 ^b	1.06 \pm 0.3 ^a	3.11 \pm 0.5 ^b

(Letters a, b represent the significant differences between raw and fortified profiles)

12.2.3. Role of ions on SGR

The role of ions in the growth of *Gracilaria* species have not been reported to date. Most early research focused on ion transport and ionic relations between the major ions between seaweed and water (Gutknecht, 1965; 1966; MacRobbie, 1962; Reed & Collins, 1980; Ritchie & Larkum, 1984a; b). The difference in SGR and net yield observed in different water types could be due to variation in the concentration of monovalent ions *viz.* Na⁺ and K⁺ or divalent ions (Ca²⁺, Mg²⁺ and Fe²⁺) or a trivalent ion (Fe³⁺). Monovalent, divalent and trivalent ions are not known to be limiting agents of seaweed growth in the marine environment as compared to N and P but their uptake rate is associated with osmoregulatory processes and efficiency in seaweeds (Reed, 1990). Therefore, the correlations between major ions (Na⁺, K⁺, Ca²⁺ and Mg²⁺), N, P and SGR was determined for raw and fortified profiles of ISW at both salinities tested (Table 12.4). In addition, to investigate the effect of one ion

on the uptake or movement of other ion(s) resulting in differences in SGR, correlations between major tissue elements Na^+ , K^+ , Ca^{2+} , Mg^{2+} , N and P with each other was also determined for raw and fortified ISW at both salinities (Table 12.5).

Table 12.4: Regression equation for the strongest correlations between major tissue elements with specific growth rate (SGR) in raw and fortified profiles at salinities 25 and 35 ppt

Factors (x, y)	Regression equation	R²
Raw profile at 35 ppt		
SGR, Na	$y = 2.52 x + 1.7$	+0.5406
Raw profile at 25 ppt		
SGR, N	$y = 0.836 x + 2.7$	+0.6897
SGR, P	$y = -0.16 x + 0.3$	-0.8330
SGR, Na	$y = -2.74 x + 3.5$	-0.6944
SGR, Fe	$y = -0.05 x + 0.05$	-0.6396
SGR, S	$y = -1.34 x + 2.5$	-0.7421
Fortified profile at 35 ppt		
SGR, N	$y = -5.72 x + 22.8$	-0.8640
SGR, Ca	$y = -12.87 x + 12.8$	-0.9482
SGR, Fe	$y = -0.17 x + 0.18$	-0.9062
SGR, Mg	$y = -1.42 x + 2.6$	-0.9678
SGR, Na	$y = -11.8 x + 16.04$	-0.8729
Fortified profile at 25 ppt		
SGR, P	$y = -0.23 x + 0.34$	-0.8441

Table 12.5: Regression equation for the strongest correlations between major tissue elements with each other in raw and fortified profiles at salinities 25 and 35 ppt

Factors (x, y)	Regression equation	R²
Raw profile at 35 ppt		
N, Ca ²⁺	y = 0.047 x – 0.3	+0.8006
P, K ⁺	y = -51.1 x + 24.0	-0.9596
P, Mg ²⁺	y = 5.55 x – 0.23	+0.9259
Na ⁺ , K ⁺	y = -2.1 x + 14.5	-0.5848
K ⁺ , Mg ²⁺	y = -0.11 x + 2.4	-0.9918
Raw profile at 25 ppt		
N, P	y = -0.18 x + 0.79	-0.9608
N, Na ⁺	y = -2.75 x + 10.7	-0.7076
N, Ca ²⁺	y = 5.75 x -15.4	+0.8546
P, Na ⁺	y = 16.67 x – 1.9	+0.8285
P, Ca ²⁺	y = -29.2 x + 9.5	-0.7033
K ⁺ , Mg ²⁺	y = 0.67 x – 5.5	+0.9697
Fortified profile at 35 ppt		
N, K ⁺	y = 0.43 x – 0.7	+0.7759
N, Ca ²⁺	y = 2.11 x – 35.9	+0.9599
N, Mg ²⁺	y = 0.23 x -2.7	+0.9513
Fortified profile at 25 ppt		
N, Na ⁺	y = 0.05 x + 2.5	+0.6606

The SGR of *G. cliftonii* is either directly or indirectly related to the concentration of different ions and their physiological roles are discussed below.

12.2.3.1. Sodium

Sodium in seaweeds is required for enzyme activation and water balance and has a major role in osmoregulation (Lobban & Harrison, 1994). In addition, it is an important component of Na/K ATPase activity and energy transfer in seaweeds. Tissue [Na⁺] showed positive correlation with SGR, [Ca²⁺], [Mg²⁺] and a negative correlation with [K⁺] in raw profiles at 35 ppt. However, in raw profiles at 25 ppt, tissue [Na⁺] was negatively correlated to SGR. In addition, tissue [Na⁺] in fortified

profiles at 35 ppt showed strong negative correlation with SGR but no correlations were observed for $[\text{Na}^+]$ at 25 ppt. The correlation of $[\text{Na}^+]$ with SGR indicates that there is regulation of Na^+ by *G. cliftonii* in altered ionic profiles, which might be affecting the growth rates.

12.2.3.2. Potassium

Potassium in seaweeds is not a limiting nutrient but is required for osmoregulation, pH control, protein formation and stability (Lobban & Harrison, 1994). It was observed that the tissue $[\text{K}^+]$ was constant in different profiles indicating that *G. cliftonii* tends to maintain an equilibrium of this ion (1965). Tissue $[\text{K}^+]$ showed strong negative correlation with $[\text{P}]$ and $[\text{Mg}^{2+}]$ in raw profiles at 35 ppt and a positive correlation with $[\text{Mg}^{2+}]$ at 25 ppt. This indicates that the lower growth rates in raw profiles at 35 ppt could be due to the regulation of K^+ , which might have suppressed P uptake. In fortified profiles at 35 ppt, it showed positive correlation with $[\text{N}]$ but no relations at 25 ppt indicating that K^+ could have resulted in absorption of N, thus resulting in higher growth rates. In addition, K^+ is required for nitrogen absorption and storage in seaweed and might have enhanced nutrient absorption, thus increasing the SGR (Lobban & Harrison, 1994).

12.2.3.3. Calcium

Calcium in seaweeds is reported to be required for structure and acts as a cofactor in ion transport (Lobban & Harrison, 1994). However, the role of Ca^{2+} in seaweeds has not been extensively studied and no report suggests this ion affects growth rates. Tissue $[\text{Ca}^{2+}]$ showed strong positive correlation with $[\text{N}]$ in raw and fortified profiles at 35 ppt. In addition, $[\text{Ca}^{2+}]$ showed positive correlation with $[\text{N}]$ while negative correlation with $[\text{P}]$ in a raw profile at 25 ppt. This indicates that the absorption of $[\text{Ca}^{2+}]$ by the tissue might have promoted the uptake of N and thus resulted in growth. In addition, the high growth rates observed in fortified profiles could be due to the combined effect of Ca^{2+} and K^+ as they both were positively correlated with the $[\text{N}]$ in *G. cliftonii*. N is assimilated at a higher rate by seaweeds under altered ionic compositions and ionic stressors (Aderhold *et al.*, 1996; Caliceti *et al.*, 2002; Davis *et al.*, 2000; Muse *et al.*, 1999). The enhanced growth of *G. cliftonii* in ISW could be due to the required absorption of N facilitated by high Ca^{2+} levels present in ISW.

12.2.3.4. Magnesium

Magnesium like calcium has also been reported as a cofactor in ion transport but forms a part of the photosynthetic component as well. It is also required for enzyme activation and ribosome stability but like Ca^{2+} has not been reported to play any role in the growth of seaweeds (Lobban & Harrison, 1994). Tissue $[\text{Mg}^{2+}]$ showed strong positive correlation with $[\text{P}]$ but was negatively correlated with N-P ratio in raw profiles at 35 ppt. This indicates that Mg might have facilitated P uptake but suppressed the overall uptake of N and P, which would have resulted in lower growth rates. As discussed earlier certain N-P ratios in nutrient media promotes growth but alteration in their proportion results in decreased growth rates (Navarro-Angulo & Robledo, 1999). However, in fortified profiles at 35 ppt, $[\text{Mg}^{2+}]$ showed strong positive correlation with $[\text{N}]$. The higher growth rates observed could be again due to the absorption of N promoted by increase in $[\text{Mg}^{2+}]$ available in ISW.

The overall correlation between major ions indicates that they might not be directly responsible for increase in growth rates but are required for nutrient absorption and may indirectly influence growth in the long run. The high growth rates in K^+ fortified water types at 35 ppt could be explained by the positive correlations of Na^+ , K^+ , Ca^{2+} and Mg^{2+} to the nitrogen content which would have assisted in nutrient absorption. It is reported that the presence of Ca^{2+} in the medium is necessary for K^+ retention by seaweeds in diluted seawater. Ca^{2+} could not be shown to participate directly in K^+ uptake and Na^+ excretion, but is involved in the maintenance of membrane structure (Eppley, 1958b). The resistance of marine algae to hypotonic solutions increases with the $[\text{Ca}^{2+}]$ of the medium (Eppley, 1958a) and is correlated with the fact that Ca^{2+} deficiency causes rapid loss of potassium from the cells (Eppley, 1958a; b).

12.3. PHYSICOCHEMICAL PROPERTIES

Difference in nutrient sources significantly influenced water retention capacity (WRC) and oil retention capacity (ORC) of *G. cliftonii* in both water types (Table 12.6). The WRC and ORC of *G. cliftonii* with nutrient supplementation were significantly higher ($p < 0.05$) than with commercial nutrient media in OW and ISW. There has been no study conducted on the influence of culture conditions particularly in ISW, on the physicochemical properties of any *Gracilaria* species. Therefore, the difference in ORC in ISW cannot be explained in the light of existing information.

However, the difference in nutrient supply along with ISW ionic profile would have altered the internal structure of *G. cliftonii* and might have provided sufficient space for retaining water and oil. In addition, this could be due to the change in chemical structure of polysaccharides due to the addition of nutrient. Roëhrig (1988) reported that physicochemical properties depend on the chemical structure of the constituent polysaccharides which can be altered due to change in external conditions. It could also be related to the hydrophilic nature of the charged polysaccharides of soluble dietary fibres (agar). However, the ORC for different seaweeds are difficult to compare with each other, because they depend on the experimental conditions (temperature, time, centrifugation) and sample preparation (McConnell *et al.*, 1974).

Table 12.6: Water retention capacity (WRC) and oil retention capacity (ORC) of *Gracilaria cliftonii* (Mean \pm S.E.) in ocean water (OW) and inland saline water (ISW) with commercial nutrient media and N-P-K supplementation

NUTRIENT	WRC (g/g)		ORC (g/g)	
	OW	ISW	OW	ISW
Commercial Media	5.9 \pm 0.2 ^a	6.0 \pm 0.3 ^a	2.7 \pm 0.2 ^a	2.9 \pm 0.1 ^a
N-P-K Supplementation	7.5 \pm 0.5 ^b	7.5 \pm 0.5 ^b	4.0 \pm 0.5 ^b	4.5 \pm 0.8 ^b

(Letters a, b represent the significant differences between nutrients)

12.4. AGAR YIELD

12.4.1. Effect of Nutrients

Different nutrient media and supplementation significantly influenced the agar yield from *G. cliftonii* (Table 12.7). Agar yield from *G. cliftonii* cultured with nutrient media was significantly higher than with nutrient supplementation in OW and ISW. It has been reported that limiting concentrations of phosphate under a high nitrogen feeding regime for *Gracilaria* species result in a higher agar yield than under high nitrogen and high phosphate concentrations (Lewis & Hanisak, 1996) which is in agreement with the present findings. In addition, higher agar yield with the nutrient media could be due to the alteration of agar structure by variable nutrient composition. It has been observed that agar isolated from *G. verrucosa* cultured under nitrogen limited conditions showed large non-polar components (ethanol

soluble), minor amounts of methylation and presence of starch, whereas nitrogen supplemented cultures produced more polar agar (hot water soluble) with little starch (Chiles *et al.*, 1989). Agar properties like gel strength, melting point, gelling temperature and sulphate content from *G. cliftonii* were not influenced ($p>0.05$) by nutrient supplementations.

Table 12.7: Agar yield (Mean \pm S.E.) from *Gracilaria cliftonii* in ocean water (OW) and inland saline water (ISW) with commercial nutrient media and N-P-K supplementation

NUTRIENT	AGAR YIELD (%db)	
	OW	ISW
Commercial Media	52.2 ± 3^a	50.7 ± 3^a
N-P-K Supplementation	27.5 ± 3^b	30.7 ± 3^b

(Letters a, b represent the significant differences between nutrients)

12.4.2. Effect of ionic profiles of ISW

In the present study, the agar yield of *G. cliftonii* from natural populations was higher than the previously reported value of 52 %db (Byrne *et al.*, 2002) but overall was not influenced by the fortification of ISW and OW mixtures at 25 and 35 ppt (Table 12.8).

Table 12.8: Agar yield (Mean \pm S.E.) from *Gracilaria cliftonii* in raw and fortified ISW profiles at both salinities

SALINITY	AGAR YIELD (%db)	
	Raw	Fortified
35 ppt	55.3 ± 2^a	55.4 ± 3^a
25 ppt	52.7 ± 1^a	55.4 ± 2^a

(Letters a, b represent the significant differences between raw and fortified profiles)

Agar yield however, showed strong correlation with the tissue elements and other properties at both salinities in raw and fortified ionic profiles of ISW (Table 12.9) which might be responsible for the alteration of other agar properties. Agar yield showed negative correlation with tissue Na^+ in raw profiles at 35 ppt indicating that there could be alteration of structure of agar with increasing Na^+ as agar contains

phosphate and sulphate groups which bind easily with Na^+ . In addition, the positive correlation of agar yield with tissue P and S in fortified profiles at both salinities indicates the accumulation of these elements in the agar structure as phosphates and sulphates form the major part of the agar structure (Andriamanananatony *et al.*, 2007; Craigie & Jurgens, 1989; Duckworth & Yaphe, 1971). This in turn affected the other properties of agar

Table 12.9: Regression equation for the strongest correlations between major tissue elements with agar yield in raw and fortified ISW profiles at salinities 25 and 35 ppt

Factors (x, y)	Regression equation	R^2
Raw profile at 35 ppt		
Na^+ , Agar yield	$y = -2.21 x + 60.7$	- 0.6566
Raw profile at 25 ppt		
No correlations		
Fortified profile at 35 ppt		
P, Agar yield	$y = 228 x + 8.7$	+ 0.9779
S, Agar yield	$y = 27.08 x - 2.555$	+ 0.7560
Fortified profile at 25 ppt		
P, Agar yield	$y = 123.05 x + 30.4$	+ 0.7543
S, Agar yield	$y = 18.99 x + 14.6$	+ 0.9900

12.4.3. Effect of alkali treatment of life stages

It was demonstrated in Chapter 8 that agar yield is a function of life stages of *G. cliftonii*. In addition, the alkali treatment of these life stages of *G. cliftonii* resulted in decrease in agar yield with or without heating time. Table 12.10 shows the correlation between alkali concentrations and agar yield. Agar yield was negatively correlated with alkali concentration for all the life stages with or without heating. This could be due to agar diffusion and/or agar biosynthesis during the alkali treatment process (Freile-Pelegrin & Robledo, 1997b).

Table 12.10: Regression equation for the strongest correlations between alkali concentration (AC) and agar yield for alkali treatment of life stages of *Gracilaria cliftonii* with or without heating

Factors (x, y)	Regression equation	R ²
Heating time = 0 h		
Carposporophyte		
AC-agar yield	$y = -945x + 51.70$	-0.8273
Tetrasporophyte		
AC-agar yield	$y = -9200x + 55$	-0.834
Vegetative		
AC-agar yield	$y = -8360.3x + 48.1$	-0.9793
Heating time = 1 h		
Carposporophyte		
AC-agar yield	$y = -1677.5x + 15$	-0.9356
Tetrasporophyte		
AC-agar yield	$y = -1402.3x + 15.3$	-0.909
Vegetative		
AC-agar yield	$y = 5311.1x + 4.8$	0.9953

12.4.4. Effect of modification of alkali treatment

In the first step of modification of the alkali treatment process, low alkali concentrations were used for treating *G. cliftonii* samples with varying soaking and heating times. Different soaking times at alkali concentrations of 0-1% significantly affected the agar yield from *G. cliftonii* (Chapter 9). However, increasing the heating time in the alkali treatment of *G. cliftonii* did not influence the agar yield (Table 12.11). The modification of the alkali treatment of *G. cliftonii* resulted in significant agar loss. It has been reported the alkali treatment of *Gracilaria* species results in 50-60 % of the agar loss (Buriyo & Kivaisi, 2003) in the process but improves the properties (Freile-Pelegrin & Robledo, 1997b; Li *et al.*, 2008; Nishinari & Watase, 1983). The introduction of alkali treatment was not the only factor resulting in loss in agar as increasing the heating time with different soaking times in treatment significantly increased the agar loss (Chapter 9).

Table 12.11: Agar yield (Mean \pm S.E.) from *Gracilaria cliftonii* at different alkali concentrations with heating times of 1 and 2 h, irrespective of soaking time

ALKALI CONCENTRATIONS	AGAR YIELD (%db)	
	Heating time of 1 h	Heating time of 2 h
0.0 %	25.4 ^a	26.9 ^a
0.3 %	17.0 ^a	19.5 ^a
0.5 %	15.0 ^a	16.4 ^a
1.0 %	14.6 ^a	19.1 ^a

(Letters a, b represent the significant differences between heating time)

The second modification step of the alkali treatment process included higher concentrations of alkali (1-5 %) and variable heating temperatures. It was demonstrated that the increase in alkali concentration of up to 5 % at different temperatures also decreases the agar yield and results in significant agar losses (Chapter 10). In addition, agar yield with alkali treatment was significantly lower than without alkali treatment. Comparing the agar yield and properties of low and high alkali concentrations with a soaking time of 3 h, heating time of 1 h and heating temperature 70 °C, it was observed that there was no significant difference in the yield with different concentrations of alkali (Table 12.12).

Table 12.12: Agar yield (Mean \pm S.E.) from *Gracilaria cliftonii* at low and high alkali concentrations with process variables, soaking time of 3 h, heating time of 1 h and heating temperature of 70 °C

ALKALI CONCENTRATIONS	AGAR YIELD (% db)
Low	20.0 \pm 1.3 ^a
High	18.8 \pm 2.0 ^a

(Letters a, b represent the significant differences between low and high alkali concentrations)

12.5. GEL STRENGTH

12.5.1. Effect of Nutrients

Nutrient media and supplementation did not influence the gel strength of the agar in OW and ISW but gel strength was significantly lower from these media than from natural populations (Table 12.13).

Table 12.13: Gel strength (Mean \pm S.E.) of agar from *Gracilaria cliftonii* in ocean water (OW) and inland saline water (ISW) with commercial nutrient media and N-P-K supplementation

NUTRIENT	GEL STRENGTH (g.cm ⁻²)	
	OW	ISW
Commercial Media	110.9 \pm 1 ^a	114.5 \pm 3 ^a
N-P-K Supplementation	117.1 \pm 1 ^a	118.4 \pm 1 ^a

(Letters a, b represent the significant differences between nutrients)

12.5.2. Effect of Ionic profiles

Gel strength of agar from *G. cliftonii* in raw ionic profiles at 25 ppt was significantly higher than in fortified ionic profiles (Table 12.14).

Table 12.14: Gel strength of agar (Mean \pm S.E.) from *Gracilaria cliftonii* in raw and fortified ISW profiles at both salinities

SALINITY	GEL STRENGTH (g.cm ⁻²)	
	Raw	Fortified
35 ppt	139.5 \pm 10 ^a	125.1 \pm 3 ^b
25 ppt	150.4 \pm 10 ^a	118.3 \pm 2 ^b

(Letters a, b represents the significant differences between raw and fortified profiles)

The decrease in the gel strength in fortified ionic profiles could be due to changes in agar solubility and/or agar biosynthesis along with structural changes due to the ion regulation under different ionic profiles of ISW (Craigie & Jurgens, 1989; Duckworth & Yaphe, 1971; Lahaye & Yaphe, 1988). Increase in [P] with supplementation in culture might have affected the structure of agar in culture conditions and in turn decreased the gel strength as the polysaccharides contain a phosphate group responsible for the gelling mechanism. In addition, the correlations

obtained between tissue element concentrations provide a better understanding of the factors influencing the gel strength of agar (Table 12.15). The strong negative correlation of gel strength with Mg^{2+} and K^{+} in raw profiles at 25 ppt indicates that the binding nature of divalent and monovalent ions could have possibly altered the structure of agar. The gelling mechanism of agar is dependent on the location of phosphate sulphate and the methoxyl group, which could easily be bound with monovalent and divalent cations. No evidence of such a mechanism has been reported in literature for monovalent cations, however Mg^{2+} is reported to be important in altering the structure by binding the charges of polysaccharide chains to one another as it is a divalent cation (Lobban & Harrison, 1994). In addition, the low gel strength of agar in the fortified profile at 35 ppt could be due to the positive correlation between tissues [S] and gel strength. It is well documented that sulphate and its location in the agar structure significantly affects the gel strength of agar (Andriamanananatnio *et al.*, 2007; Armisen & Galatas, 1987; Craigie & Jurgens, 1989; Duckworth & Yaphe, 1971; Freile-Pelegrin & Robledo, 1997b; Lahaye & Rochas, 1991; Lahaye & Yaphe, 1988). The accumulation of S by *G. cliftonii* in culture would have altered the sulphate content of agar but no correlation between S and the sulphate content of agar was observed.

Table 12.15: Regression equation for the strongest correlations between major tissue elements with gel strength in raw and fortified ISW profiles at salinities 25 and 35 ppt

Factors (x, y)	Regression equation	R²
Raw profile at 35 ppt		
Ca, Gel strength	$y = 467 x - 129$	+0.9700
Raw profile at 25 ppt		
Mg, Gel strength	$y = -214.2 x + 380$	-0.8848
K, Gel strength	$y = -145.67 x + 1592$	-0.8929
Fortified profile at 35 ppt		
S, Gel strength	$y = 63.07 x - 9.9$	+0.7258
Fortified profile at 25 ppt		
No correlations		

12.5.3. Effect of alkali treatment of life stages

Gel strength of agar varied as a function of the life stages of *G. cliftonii*. In addition, the alkali treatment of reproductive and vegetative stages with variable soaking and heating time had no significant effect on the gel strength. It has been reported that alkali treatment of *Gracilaria* species is required to produce agar with a high gel strength (Armisen, 1995; Armisen & Galatas, 1987). In addition, alkali treatment of *Gracilaria* species is due to its high sulphate content and alkali converts L-galactose-6-sulphate to 3,6-anhydro-L-galactose (Duckworth & Yaphe, 1971), which is responsible for the enhancement of the gel forming ability (Freile-Pelegrin & Robledo, 1997b). However, the alkali treatment of different life stages in this study resulted in the lowering of gel strength. In addition, alkali concentrations showed strong negative correlation with the gel strength of agar from different life stages without heating (Table 12.16). A positive correlation was observed between gel strength and alkali concentrations for carposporophyte stages with 1 h heating time. This indicates that heating during the alkali treatment of *G. cliftonii* is required to increase the gel strength of agar.

Table 12.16: Regression equation for the strongest correlations between alkali concentration (AC) and gel strength of agar for alkali treatment of life stages of *G. cliftonii* with or without heating

Factors (x, y)	Regression equation	R ²
Heating time = 0 h		
Carposporophyte		
AC-gel strength	$y = -6131 x + 163$	-0.9851
Tetrasporophyte		
AC-gel strength	$y = -3821 x + 155$	-0.6688
Vegetative		
AC-gel strength	$y = -6631.6 x + 183.6$	-0.8824
Heating time = 1 h		
Carposporophyte		
AC-gel strength	$y = 6076 x + 132.6$	+0.9476
Tetrasporophyte		
AC-gel strength	-	-
Vegetative		
AC-gel strength	-	-

12.5.4. Effect of modification of alkali treatment

It was demonstrated in Chapter 9 that alkali treatment of *G. cliftonii* with variable soaking and heating times at low alkali concentrations had a detrimental effect on the gel strength of agar. Different soaking times significantly influenced the gel strength of agar but different heating times during alkali treatment had no influence on the gel strength (Table 12.17).

Table 12.17: Gel strength of agar (Mean \pm S.E.) from *Gracilaria cliftonii* at different alkali concentrations with heating times of 1 and 2 h, irrespective of soaking time

ALKALI CONCENTRATION	GEL STRENGTH (g.cm ⁻²)	
	Heating time of 1 h	Heating time of 2 h
0.0 %	137.3	136.1
0.3 %	130.6	132.3
0.5 %	135.5	120.1
1.0 %	118.2	117.8

(Letters a, b represent the significant differences between heating time)

It was demonstrated in Chapter 10 that an increase in alkali concentration to 3 and 5 % with a heating temperature of 60 °C significantly improves the gel strength of agar. However, at 70 °C there was no significant difference between the gel strength of agar with low or high alkali concentrations (Table 12.18).

Table 12.18: Gel strength of agar (Mean \pm S.E.) from *Gracilaria cliftonii* at low and high alkali concentrations with process variables, soaking time of 3 h, heating time of 1 h and heating temperature of 70 °C

ALKALI CONCENTRATION	GEL STRENGTH (g.cm ⁻²)
Low	124.3 \pm 3.9 ^a
High	147.2 \pm 7.5 ^a

(Letters a, b represent the significant differences between low and high alkali concentrations)

12.6. MELTING POINT AND GELLING TEMPERATURE

12.6.1. Effect of Nutrients

Nutrient supplementation had no influence on the melting point and gelling temperature of agar in OW and ISW (Table 12.19).

Table 12.19: Melting point and gelling temperature of agar (Mean \pm S.E.) from *Gracilaria cliftonii* in ocean water (OW) and inland saline water (ISW) with commercial nutrient media and N-P-K supplementation

NUTRIENT	MELTING POINT		GELLING	
	(°C)		TEMPERATURE (°C)	
	OW	ISW	OW	ISW
Commercial nutrient Media	83.8 \pm 1 ^a	83.2 \pm 2 ^a	33.8 \pm 1 ^a	34.1 \pm 1 ^a
N-P-K Supplementation	86.1 \pm 1 ^a	85.8 \pm 2 ^a	33.6 \pm 1 ^a	34.7 \pm 1 ^a

(Letters a, b represent the significant differences between nutrients)

12.6.2. Effect of fortification of ionic profiles

Fortification of ionic profiles had no influence on the melting point and gelling temperature of agar at both salinities (Table 12.20).

Table 12.20: Melting point and gelling temperature of agar (Mean \pm S.E.) from *Gracilaria cliftonii* in raw and fortified ISW profiles at both salinities

SALINITY	MELTING POINT (°C)		GELLING TEMPERATURE (°C)	
	Raw	Fortified	Raw	Fortified
35 ppt	84.5 \pm 1.9 ^a	82.2 \pm 1.4 ^a	35.6 \pm 0.5 ^a	33.2 \pm 0.7 ^a
25 ppt	83.9 \pm 1.5 ^a	82.4 \pm 1.0 ^a	35.3 \pm 0.4 ^a	33.9 \pm 0.8 ^a

(Letters a, b represent the significant differences between raw and fortified profiles)

12.6.3. Effect of alkali treatment of life stages

It was demonstrated in Chapter 8 that the melting point and gelling temperature of agar are a function of the life stages of *G. cliftonii*. In addition, the alkali treatment of life stages of *G. cliftonii* had no significant influence on the melting point and gelling temperature of agar. However, few negative correlations were observed for different life stages between alkali concentration and melting point or gelling temperature (Table 12.21).

Table 12.21: Regression equations for the strongest correlation between alkali concentration (AC) and melting point/gelling temperature of agar for alkali treatment of life stages of *Gracilaria cliftonii* with or without heating

Factors (x, y)	Regression equation	R ²
Heating time = 0 h		
Carposporophyte		
AC - melting point	$y = -1399.9 x + 89.3$	-0.9933
AC – gelling temperature	-	-
Tetrasporophyte		
AC - melting point	$y = -726.3 x + 87.6$	-0.9741
AC – gelling temperature	-	-
Vegetative		
AC - melting point	-	-
AC – gelling temperature	$y = -1015.7 x + 37.8$	-0.994
Heating time = 1 h		
Carposporophyte		
AC - melting point	$y = -341.1 x + 87.5$	-0.9138
AC – gelling temperature	$y = -219.2 x + 33.7$	-0.922
Tetrasporophyte		
AC - melting point	$y = -665.7 x + 84.6$	-0.9905
AC – gelling temperature	$y = -336 x + 32.9$	-0.9954
Vegetative		
AC - melting point	-	-
AC - melting point	-	-

12.6.4. Effect of modification of alkali treatment

It was demonstrated in Chapter 9 that soaking time and not heating time in alkali treatment was significantly influencing the melting point and gelling temperature of agar. No significant difference was observed in the melting point and gelling temperature of agar at different heating times irrespective of soaking. However, with 1 % alkali treatment melting point of agar significantly decreased as compared to other alkali concentrations at both heating times (Table 12.22). Overall alkali treatment with variable soaking and heating times had a detrimental effect on melting point and gelling temperature. This could be due to the alteration of agar structure caused by the introduction of alkali. Gelling temperature is dependent on methoxyl content (Guiseley, 1970; Rebello *et al.*, 1997) while melting point is dependent on pyruvic acid content (Young *et al.*, 1971) both of which get altered during alkali treatment (Andriamanananatony *et al.*, 2007; Freile-Pelegrin & Robledo, 1997b; Nishinari & Watase, 1983).

Table 12.22: Melting point and gelling temperature of agar (Mean \pm S.E.) from *Gracilaria cliftonii* at different alkali concentrations with heating times of 1 and 2 h, irrespective of soaking time

Alkali concentration	Melting Point (°C)		Gelling Temperature (°C)	
	1 h Heating	2 h Heating	1 h Heating	2 h Heating
0.0 %	81.2 ^a	81.3 ^a	32.2 ^a	32.0 ^a
0.3 %	80.4 ^a	80.8 ^a	31.9 ^a	31.6 ^a
0.5 %	80.2 ^a	80.1 ^a	31.5 ^a	31.2 ^a
1.0 %	78.5 ^a	75.5 ^a	31.2 ^a	31.2 ^a

(Letters a, b represent the significant differences between heating time)

Modification of alkali treatment with higher alkali concentrations at different temperatures did not influence the melting point and gelling temperature of agar and was similar to natural populations (Chapter 10). Comparing the melting point and gelling temperature of agar of low and high alkali concentrations with a soaking time of 3 h, heating time of 1 h and heating temperature 70 °C, it was observed that both properties were significantly lower ($p < 0.05$) for alkali treatment with low alkali

concentrations than high alkali concentrations (Table12.23). The difference in melting and gelling temperature is due to change in structure as explained earlier.

Table 12.23: Melting point and gelling temperature of agar (Mean \pm S.E.) from *Gracilaria cliftonii* at low and high alkali concentrations with process variables, soaking time of 3 h, heating time of 1 h and heating temperature of 70 °C

ALKALI CONCENTRATION	MELTING POINT (°C)	GELLING TEMPERATURE (°C)
Low	82.2 \pm 1.2 ^a	30.6 \pm 0.4 ^a
High	85.2 \pm 0.5 ^b	35.1 \pm 0.4 ^b

(Letters a, b represent the significant differences between low and high alkali concentrations)

12.7. SULPHATE CONTENT

12.7.1. Effect of Nutrients

The sulphate content of agar from *G. cliftonii* cultured with nutrient media in OW was significantly higher ($p < 0.05$) than with nutrient supplementation in OW (Table12.24). This could be due to the absorption of sulphates from nutrients in the media.

Table 12.24: Sulphate content of agar (Mean \pm S.E.) from *Gracilaria cliftonii* in ocean water (OW) and inland saline water (ISW) with commercial nutrient media and N-P-K supplementation

NUTRIENT	SULPHATE CONTENT (%db)	
	OW	ISW
Commercial Media	5.0 \pm 1 ^a	5.2 \pm 1 ^a
N-P-K Supplementation	3.4 \pm 1 ^b	5.7 \pm 1 ^a

(Letters a, b represent the significant differences between nutrients)

12.7.2. Effect of fortification of ionic profiles

Fortification of ionic profiles had no influence on the sulphate content of agar at both salinities (Table 12.25).

Table 12.25: Sulphate content of agar (Mean \pm S.E.) from *Gracilaria cliftonii* in raw and fortified ISW profiles at both salinities

SALINITY	SULPHATE CONTENT (%db)	
	Raw	Fortified
35 ppt	6.3 \pm 0.9 ^a	5.1 \pm 0.4 ^b
25 ppt	6.2 \pm 0.9 ^a	5.2 \pm 0.3 ^b

(Letters a, b represent the significant differences between raw and fortified profiles)

12.7.3. Effect of alkali treatment of life stages

It was demonstrated in Chapter 8 that the sulphate content of agar is a function of the life stages of *G. cliftonii*. In addition, it was demonstrated that the sulphate content of agar decreased with the alkali treatment of different stages but this did not improve the gel strength.

12.7.4. Effect of modification of alkali treatment

Modification of the alkali treatment process did not reduce the sulphate content of agar and thus resulted in low gel strength of agar (Chapter 10). Similar sulphate content was observed at different alkali concentrations and both heating times (Table 12.26). In addition, the sulphate content of agar after alkali treatment was similar to natural populations indicating that the low concentration of alkali along with soaking and heating might not be able to convert L-galactose-6-sulphate to 3,6-anhydro-L-galactose. This could be due to the low concentrations of alkali used in the process. It has been reported that alkali treatment variables like concentration, soaking and heating time are species specific and have to be identified in order to improve the agar properties (Armisen & Galatas, 1987; Arvizu-Higuera *et al.*, 2008; Nishinari & Watase, 1983).

Table 12.26: Sulphate content of agar (Mean \pm S.E.) from *Gracilaria cliftonii* at different alkali concentrations with heating times of 1 and 2 h, irrespective of soaking time

ALKALI CONCENTRATION	SULPHATE CONTENT (%db)	
	Heating time of 1 h	Heating time of 2 h
0.0 %	6.6 ^a	6.4 ^a
0.3 %	6.4 ^a	6.3 ^a
0.5 %	6.2 ^a	6.1 ^a
1.0 %	6.2 ^a	5.6 ^a

(Letters a, b represent the significant differences between heating time)

Comparing the sulphate content of agar for low and high alkali concentrations with a soaking time of 3 h, heating time of 1 h and heating temperature 70 °C, it was observed the sulphate content was significantly reduced ($p < 0.05$) with a higher concentration of alkali as compared to a lower concentration (Table 12.27). This could be due to the conversion of L-galactose-6-sulphate to 3,6-anhydro-L-galactose and thus improved gel strength at high alkali concentrations (Freile-Pelegrin & Robledo, 1997b; Nishinari & Watase, 1983).

Table 12.27: Sulphate content of agar (Mean \pm S.E.) from *Gracilaria cliftonii* at low and high alkali concentrations with process variables, soaking time of 3 h, heating time of 1 h and heating temperature of 70 °C

ALKALI CONCENTRATION	SULPHATE CONTENT (%db)
Low	6.4 \pm 0.2 ^a
High	2.9 \pm 0.3 ^b

(Letters a, b represent the significant differences between low and high alkali concentrations)

12.8. MODIFICATION OF AGAR EXTRACTION PROCESS

It was demonstrated that alkali treatment of *G. cliftonii* with different variables decreased the agar yield significantly but improved the agar properties (Chapters 9 and 10). In addition the decrease in sulphate content did not improve the gel strength

as reported by various authors (Freile-Pelegrin & Robledo, 1997b; Nishinari & Watase, 1983). The modification of the agar extraction process by varying a few variables resulted in improvement in the agar properties without loss of agar. It was demonstrated that *G. cliftonii* samples soaked for 1 h at 30 °C with a seaweed-water ratio of 1:150 and extraction of agar in boiling water at 100 °C for 3 h resulted in high agar yield and quality. The agar yield with modification of the process was higher than from the alkali treatment process. In addition, agar properties, particularly the gel strength of agar was similar between the two processes. It was demonstrated that agar yield and properties are influenced by different variables in the agar extraction process. It also shows that agar characteristics can be manipulated in terms of soaking time and temperature, weed-water ratio, extraction time and temperature in the agar extraction process. The results provide the basis to define the agar extraction process for *G. cliftonii* to obtain maximum yield and optimum properties. The agar extraction process to be followed for optimum agar yield and quality from *G. cliftonii* is shown in Figure 12.1.

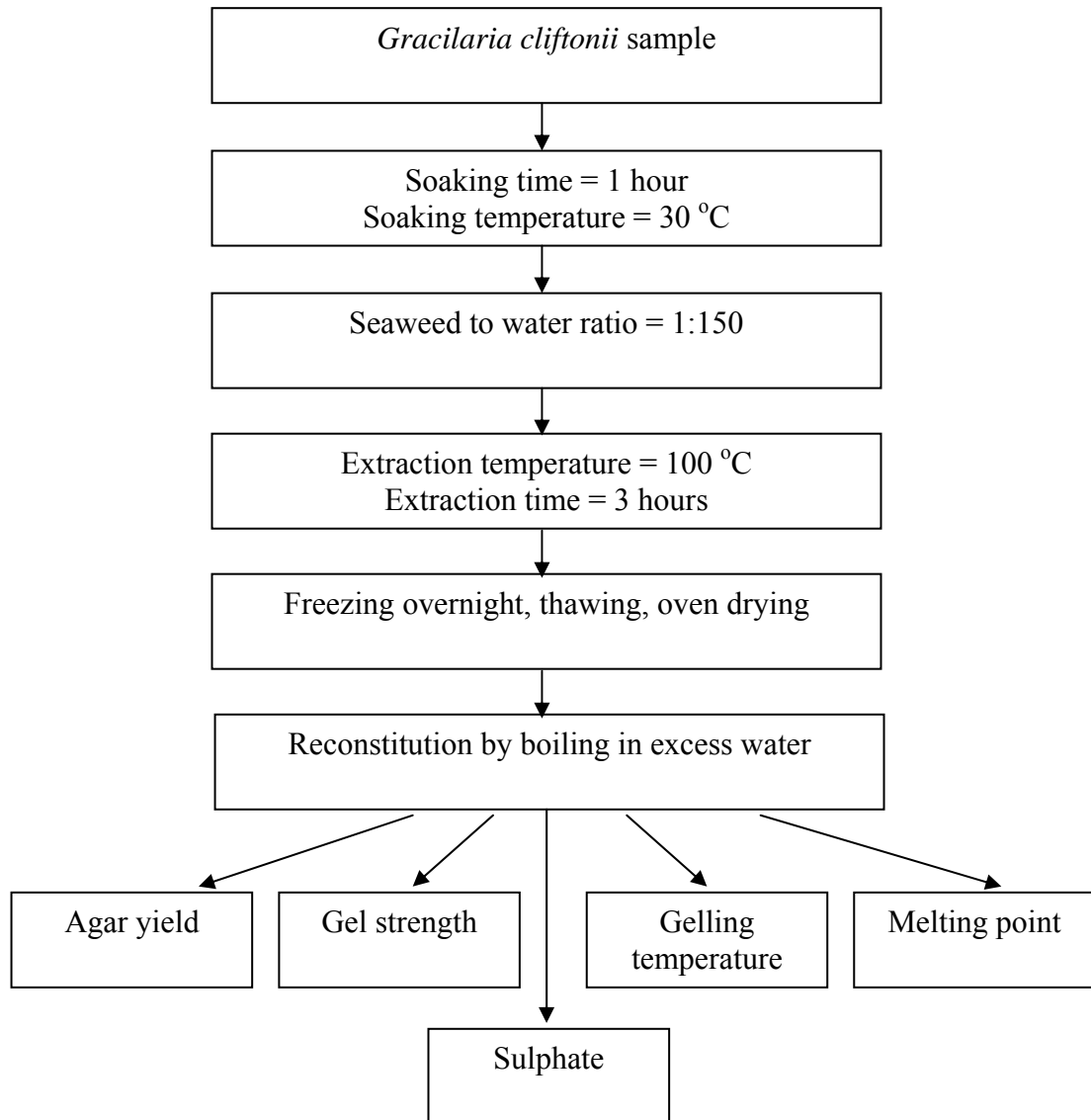


Figure 12.1: Outline of the optimum agar extraction process for *Gracilaria cliftonii* as determined from the results of the present study

12.9. CONCLUSIONS

1. *Gracilaria cliftonii* has a high potential in health food development due to its high protein and fat content. In addition, the high agar content of *G. cliftonii* demonstrates it is a source of agar production.
2. The growth of *G. cliftonii* in ISW and OW indicates its potential as a candidate species for ISW aquaculture. In addition, selection and continuous supply of nutrient media in culture conditions is important to ensure the continuous growth of *G. cliftonii*.
3. The composition of nutrients in the media is important for culturing *G. cliftonii*. Use of commercially available nutrient media results in better growth rates as compared to nutrient supplementation in the form of N-P-K. This indicates an appropriate quantity and ratio of nutrients along with vitamins and trace metal should be supplied in order to obtain higher growth rates in ISW culture.
4. Supply of appropriate nutrient media results in a high yield of *G. cliftonii* in ISW as compared to OW.
5. *G. cliftonii* can be grown in ISW at different locations and with different salinities irrespective of the ionic profile although the growth rates are very low suggesting it is a slow growing species.
6. The low $[K^+]$ of raw ISW significantly affects *G. cliftonii* growth rates but the problem can be overcome by fortifying ISW with external K^+ to yield high growth rates.
7. Bringing the ionic profiles of ISW closer to that of OW had no influence on the SGR of *G. cliftonii*. However, when K^+ alone was raised to an equivalent concentration as in OW, the SGR of *G. cliftonii* increased significantly.
8. Different nutrient media and supplementations have a significant effect on the physicochemical and agar properties of *G. cliftonii*.
9. Different ionic profiles of ISW with or without fortification also significantly affected the chemical composition, physicochemical and agar properties of *G. cliftonii*.
10. The chemical composition of *G. cliftonii* in different ionic profiles of ISW with or without fortification indicates that K^+ and Mg^{2+} are maintained at equilibrium.
11. The reproductive and vegetative stages of *G. cliftonii* have different agar yield and properties suggesting that the time of harvest and stage should be selected carefully to achieve higher agar yield.

12. Alkali treatment of *G. cliftonii* irrespective of stages, at low concentrations results in loss of agar yield and quality. However, at high alkali concentrations the quality of agar improves considerably.
13. Different alkali treatment variables significantly influence the agar characteristics and should be optimised to obtain good yield and quality.
14. Different variables in the agar extraction process significantly affect the agar yield and quality. In addition, agar yield and properties can be improved by manipulating the extraction processing variables.

12.10. RECOMMENDATIONS FOR FURTHER RESEARCH

1. Research into broader salinity profiles to investigate the range of salinity tolerance of *Gracilaria cliftonii*
2. More ecological studies are required under natural conditions to understand the life cycle and biomass increment
3. Investigate a broader range of N-P ratios as nutrient supplements for culturing *G. cliftonii*.
4. Investigate the effects of varying physical and chemical factors such as irradiance, temperature and pH on the growth rates of *G. cliftonii*.
5. Agar extraction variables like freezing time and thawing should be standardised to optimise the agar characteristics of *G. cliftonii*.
6. Agar extraction parameters should be modified depending on the desirable agar property rather than improvement of overall agar quality.
7. The high proximate composition of *G. cliftonii* especially protein should be investigated for food product development by determining amino acid profiles.
8. Seaweed residue from agar extraction which has a high protein and mineral content, should be investigated for development of aquaculture feeds.

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APPENDICES

Appendix 1

Specific growth rate and net yield of *G. cliftonii* in N-P-K supplemented ocean and inland saline water for every three weeks

Appendix 2

Heavy metal composition of *G. cliftonii* in culture in different ionic profiles

Appendix 3

Alkali treatment and agar extraction process of *Gracilaria cliftonii* life stages

Appendix 4

Modification of alkali treatment for *Gracilaria cliftonii* with low alkali concentration, soaking time and heating time

Appendix 5

Modification of alkali treatment *Gracilaria cliftonii* with high alkali concentration and heating temperature

Appendix 1

Specific growth rate and net yield of *G. cliftonii* in N-P-K supplemented ocean and inland saline water for every three weeks

Table I: Specific growth rate of *G. cliftonii* in N-P-K supplemented ocean and inland saline water for nine weeks.

Ocean Water	Weeks		
	3	6	9
Raw	0.35 ± 0.02^a	0.44 ± 0.02^a	0.42 ± 0.03^a
N:P:K::1:1:1	0.22 ± 0.02^b	0.32 ± 0.02^b	0.33 ± 0.01^b
N:P:K::2:1:1	0.21 ± 0.02^b	0.23 ± 0.02^b	0.21 ± 0.00^b
N:P:K::1:2:1	0.42 ± 0.06^a	0.44 ± 0.03^a	0.44 ± 0.00^a
N:P:K::1:1:2	0.23 ± 0.02^b	0.22 ± 0.01^a	0.19 ± 0.01^b
Inland saline water			
Raw	0.28 ± 0.03^a	0.26 ± 0.03^a	0.29 ± 0.01^a
N:P:K::1:1:1	0.27 ± 0.01^a	0.21 ± 0.01^a	0.20 ± 0.01^b
N:P:K::2:1:1	0.25 ± 0.02^a	0.22 ± 0.01^a	0.20 ± 0.01^b
N:P:K::1:2:1	0.38 ± 0.04^b	0.47 ± 0.01^b	0.52 ± 0.02^c
N:P:K::1:1:2	0.20 ± 0.01^c	0.21 ± 0.03^a	0.26 ± 0.01^a

Table II: Specific growth rate of *G. cliftonii* in N-P-K supplemented ocean and inland saline water for nine weeks.

Ocean Water	Weeks		
	3	6	9
Raw	0.98 ± 0.06^a	1.31 ± 0.07^a	1.31 ± 0.12^a
N:P:K::1:1:1	0.60 ± 0.06^b	0.95 ± 0.05^b	1.00 ± 0.04^b
N:P:K::2:1:1	0.58 ± 0.06^b	0.65 ± 0.05^c	0.63 ± 0.02^c
N:P:K::1:2:1	1.21 ± 0.17^c	1.33 ± 0.10^a	1.38 ± 0.02^a
N:P:K::1:1:2	0.65 ± 0.05^b	0.62 ± 0.05^c	0.54 ± 0.03^c
Inland saline water			
Raw	0.80 ± 0.10^a	0.75 ± 0.09^a	0.87 ± 0.05^a
N:P:K::1:1:1	$0.77 \pm 0.04^{a,c}$	0.61 ± 0.03^a	0.58 ± 0.04^b
N:P:K::2:1:1	$0.70 \pm 0.06^{a,c}$	0.62 ± 0.04^a	0.58 ± 0.03^b
N:P:K::1:2:1	1.09 ± 0.12^b	1.41 ± 0.04^b	1.67 ± 0.06^c
N:P:K::1:1:2	0.54 ± 0.04^c	0.60 ± 0.09^a	0.75 ± 0.04^a

Appendix 2

Heavy metal composition of *G. cliftonii* in culture in different ionic profiles

Table I: Heavy metal composition of *Gracilaria cliftonii* in culture in different ionic profiles (mg/kg).

Ionic profile	B	Zn	Mn	Cu
Natural population	203	89	78	4
³⁵ ISW ₀	330	311	564	16
³⁵ ISW ₃₃	319	299	249	13
³⁵ ISW ₆₆	341	291	191	15
³⁵ ISW ₁₀₀	253	292	214	13
²⁵ ISW ₀	357	307	159	17
²⁵ ISW ₃₃	299	189	159	13
²⁵ ISW ₆₆	278	240	201	14
²⁵ ISW ₁₀₀	210	210	190	11

Appendix 3

Alkali treatment and agar extraction process of *Gracilaria cliftonii* life stages

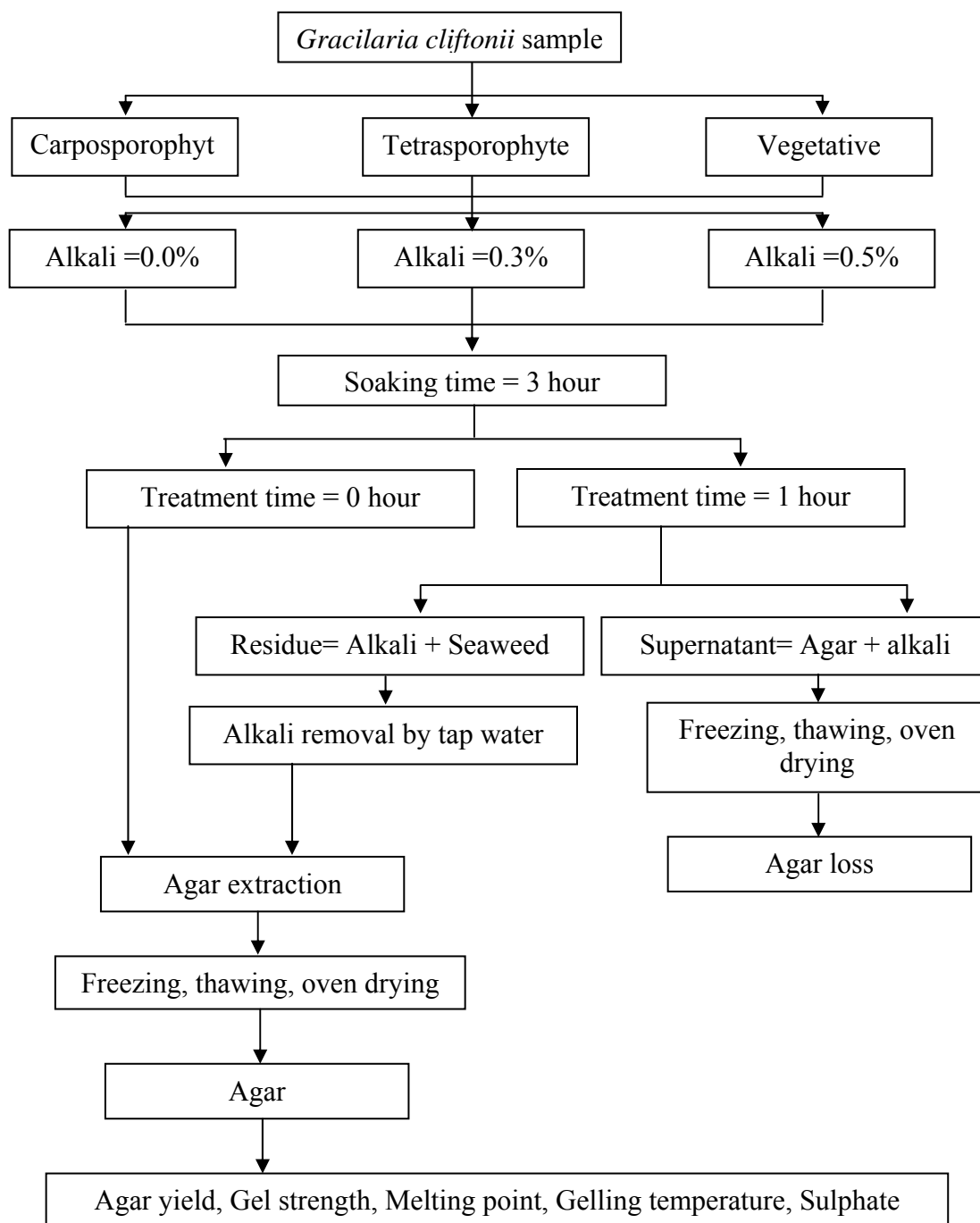


Figure A: Alkali treatment and agar extraction process of *Gracilaria cliftonii* life stages

Appendix 4

Modification of alkali treatment for *Gracilaria cliftonii* with low alkali concentration, soaking time and heating time

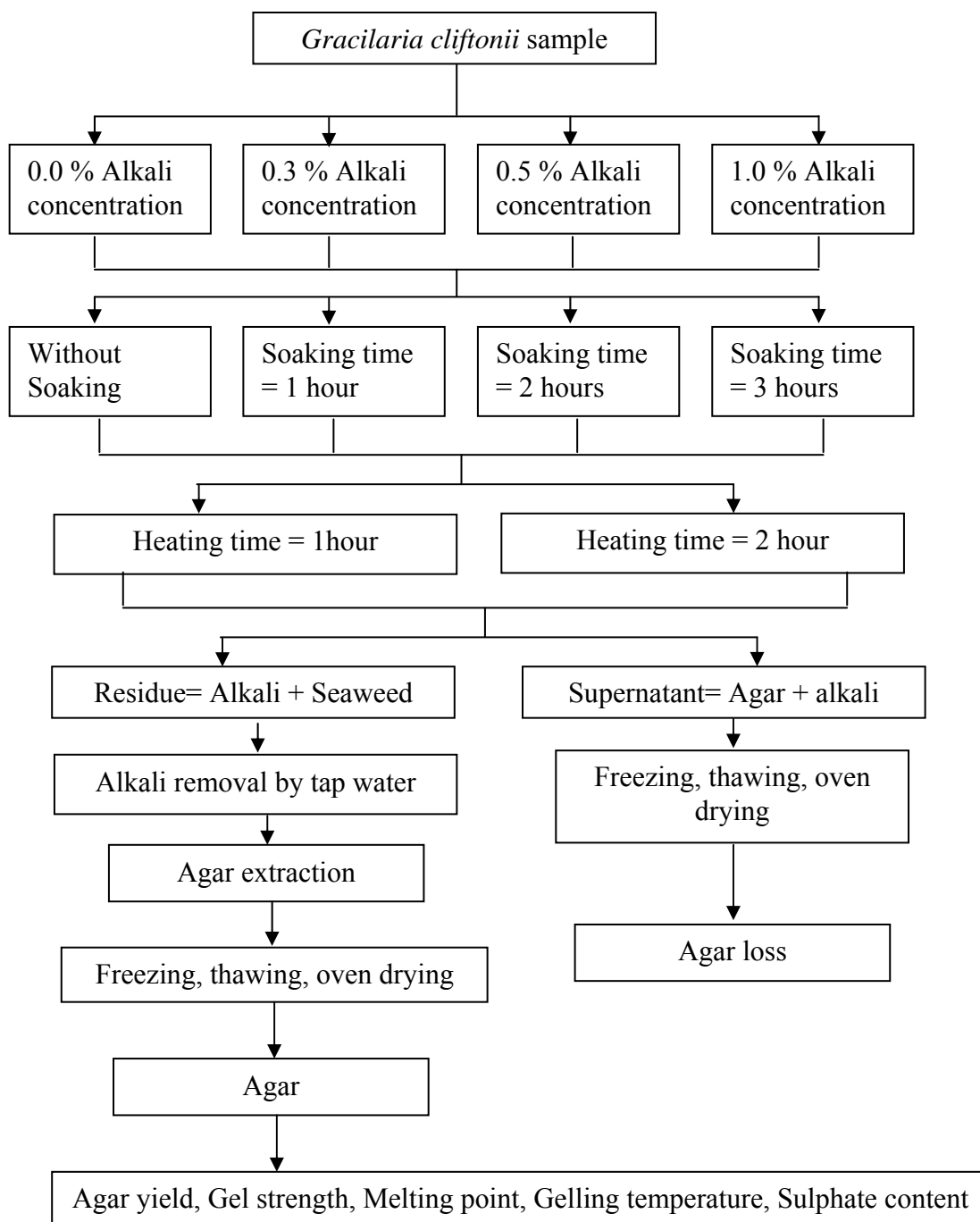


Figure A: Modification of alkali treatment for *Gracilaria cliftonii* with low alkali concentration, soaking time and heating time

Appendix 5

Modification of alkali treatment *Gracilaria cliftonii* with high alkali concentration and heating temperature

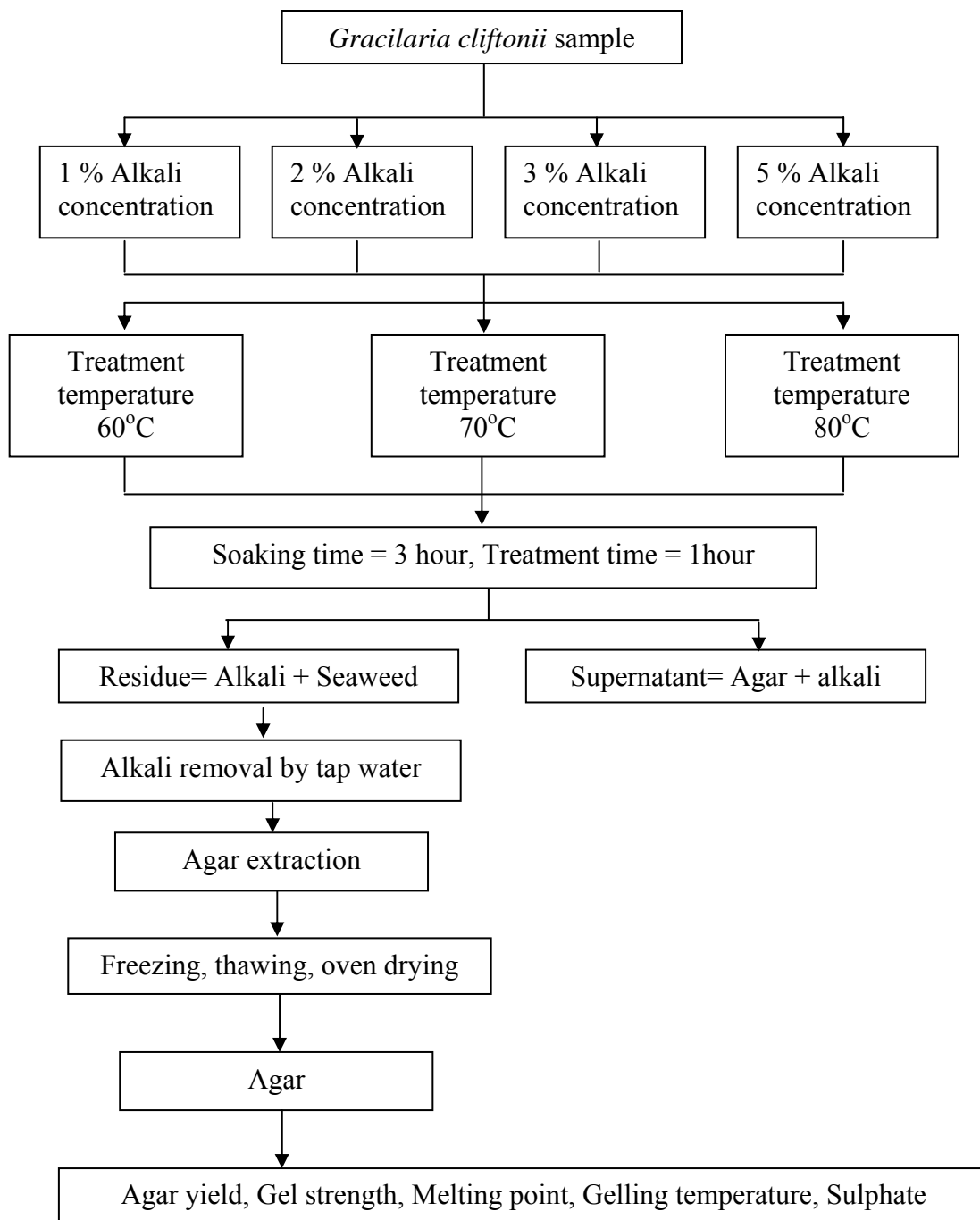


Figure A: Modification of alkali treatment of *Gracilaria cliftonii* with high alkali concentration and heating temperature